Exercise, neuropeptides and the hypothalamic regulation of appetite and energy balance

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Dedication

This dissertation is dedicated to my family, for always believing in me; and to my partner, who has been patient, loving, and supportive through this process; and finally, to my community, for providing encouragement and emotional support.

Abstract

This dissertation is focused on mechanisms involved in the central regulation of appetite, with particular focus on interventions affecting two specific hypothalamic nuclei involved in feeding and energy balance, the ventromedial hypothalamic (VMN) and paraventricular nuclei (PVN). The purpose of these investigations was to identify promising areas of intervention on brain mechanisms involved in the etiology of obesity.

First I sought to determine whether exercise dynamically modifies the brain to promote negative energy balance via altering homeostatic appetitive responses. A thorough literature review was performed, which led to the conclusion that, particularly during obesity, aerobic exercise may promote negative energy balance via paradoxically reducing caloric intake despite the increased energy demands of exercise. I then performed a literature review to investigate one particular factor which I believe has promising relevance for how exercise may alter the structure or activity of appetitive regions in the hypothalamus, brain-derived neurotrophic factor (BDNF). In the hypothalamus, BDNF and its receptor, tropomyosin-related kinase B (trkB), are extensively expressed in areas associated with feeding and metabolism, and have been demonstrated to inhibit food intake and increase energy expenditure in both the PVN and VMN, leading to negative energy balance. Furthermore, BDNF via its receptor trkB has a known role in promoting synaptic plasticity and

synaptogenesis. Since exercise has been shown to promote sustained alterations in appetite regulation toward maintenance of a leaner phenotype, I hypothesized that exercise-induced feeding reductions are associated with elevated BDNF and trkB in appetite related areas of the hypothalamus.

Using Sprague-Dawley rats, I show that over an eight-week period cumulative food intake is reduced in exercising animals compared with sedentary controls, leading to an overall negative energy balance. I report that during the early stages of exercise training, PVN BDNF is elevated in relationship to the amount of running performed by the animals. I did not observe significant changes in BDNF at the eight-week time point, suggesting that exercise may result in early plasticity changes in the PVN, which may alter the function or responsiveness of the PVN during the long-term. In addition to BDNF, I measured trkB receptor in the PVN and surrounding area. I report discovery of trkB immunoreactive fibers surrounding the PVN that have not been previously described in the literature. Quantification of the density of trkB immunoreactive fibers in animals subjected to either volitional or forced running paradigms indicated that volitional running was associated with a reduction in fiber density compared with forced exercise.

The second portion of this dissertation focuses on the effects of oxytocin in the VMN on energy balance. Oxytocin, specifically produced in the PVN, has been shown previously to be essential to maintaining energy balance. Currently,

literature related to oxytocin is focused on either hindbrain effects of oxytocin on energy balance, or relies on intra cerebroventricular injections, which provide no information about potential sites of oxytocin forebrain effects. Using site-specific VMN injections, I demonstrate for the first time that oxytocin reduces feeding and increases both activity and energy expenditure in this forebrain site. These data are relevant to understanding mechanisms by which oxytocin reduces feeding, and provides insight into the role of oxytocin in the central regulation of energy balance.

Table of Contents

List of Figures

Chapter 1
Introduction

General Introduction

This dissertation focuses on the hypothalamic regulation of energy balance, with two distinct but related goals. One is to investigate the effects of exercise on the central regulation of appetite and investigate the likelihood that these effects might be associated with changes in markers of hypothalamic plasticity. The second is to investigate whether oxytocin is a negative regulator of energy balance in the ventromedial hypothalamus. Though these investigations are seemingly unrelated, they both share the common goal of increasing our understanding of physiological mechanisms that could lead to effective therapeutics for treating obesity. In this general introduction I give a brief overview of the problem of obesity and the role of the hypothalamus in the regulation of energy balance with a specific focus on the areas most relevant to this dissertation, the ventromedial hypothalamus and the paraventricular nucleus. I also include a brief overview of oxytocin, but for the sake of avoiding redundancy I will avoid discussing brain derived neurotrophic factor and exercise in this general introduction, since both of these topics have an entire chapter devoted to discussing relevant research related to them. At the end of this general introduction I will outline each of the chapters of this dissertation.

Overview of obesity

Obesity is considered to be a major concern to public heath in the United States and has a population prevalence of between 34.9 % for adults and 16.9% for children (Ogden, Carroll et al. 2014). The obesity prevalence rates have not increased compared with data from 2003, indicating that the prevalence rates

have leveled off (Ogden, Carroll et al. 2014), however among obese individuals the category of type 3 obesity defined as a body mass index >40 has continued to rise (Sturm and Hattori 2013). Thus individuals who are obese are continuing to become more obese. Quality of life, health risks, and costs of healthcare are three reasons why it is important to understand physiological mechanisms, which may benefit in the treatment of obesity.

Susceptibility to obesity is highly heritable, as evidenced by studies of monozygotic twins. For example, in a study of 658 pairs of monozygotic twins, only 18 pairs were discordant for obesity (Pietilainen, Rissanen et al. 2004). However, physical activity has demonstrated to reduce the influence of genetic factors on obesity (Mustelin, Silventoinen et al. 2009). The inter-individual differences in physical activity and sedentary behavior are more than 50% explained by the environment and not genetics (den Hoed, Brage et al. 2013). Thus physical activity is a modifiable variable that can reduce the influence of genetics on susceptibility to obesity, given the right environmental influence. Unfortunately, the current environment in the United States, and increasingly across the globe, is one where there is an over abundance of highly palatable food and a reduced requirement for physical activity due to modern transportation. According to the American Time Use Survey, approximately 79% of full-time working Americans are employed in sedentary jobs, which combined with sleeping accounts for 15.3 hours of a 24-hour day. The remaining hours are spent engaging in sedentary behaviors (4.2 hours) or light intensity activity (3.8 hours) (Tudor-Locke, Leonardi et al. 2011). Evidence suggests that being sedentary leads to elevated food consumption compared with being moderately active. For example, those confined to complete bed-rest reportedly eat more food compared with those on bed-rest who were on a moderate daily exercise program (Bergouignan, Momken et al. 2010) or just not confined to bed-rest (Thivel, Metz et al. 2013). One of the aims of this dissertation is to investigate

some of the mechanisms contributing to the effects of activity or non-activity on appetite and adiposity. Data from rodent studies suggests that once obesity is established in a susceptible individual, weight loss and the maintenance of a lean body weight requires maintaining more of an energy deficit than would be predicted for an individual of lean body weight that has never been obese (Hill 2006; MacLean, Higgins et al. 2009). It is therefore important to gain insight into some of the central mediators regulating energy balance. When mechanisms underlying the decision of whether to move and whether to eat are understood, obesity can be effectively treated. As will be discussed in later chapters, exercise has promising effects in the treatment of obesity. After exercise, obese individuals reportedly eat less food compared with during sedentary conditions, but have no changes in their perceived satiety (Thivel, Isacco et al. 2011; Thivel, Isacco et al. 2011; Thivel, Isacco et al. 2012; Guelfi, Donges et al. 2013; Holmstrup, Fairchild et al. 2013; Knudsen, Karstoft et al. 2014), and in some cases hunger was also reduced by exercise (Holmstrup, Fairchild et al. 2013). Thus exercise has the capacity to modify unconscious behaviors leading to reduced obesity. Individuals were eating less without the sense of deprivation that comes during dieting. To better understand the mechanisms underlying the promising effects of exercise in the treatment of obesity, we looked to the hypothalamus.

Hypothalamic regulation of energy balance

The hypothalamus contains populations of cells involved in the homeostatic regulation of energy balance, such as energy intake, thermogenesis and physical activity. Of more than 40 distinct hypothalamic nuclei, certain key areas are heavily researched in the context of energy balance and appetite, including the arcuate nucleus (ARC), ventromedial hypothalamus (VMN), paraventricular nucleus (PVN) and lateral hypothalamic area (LH) (Meister 2007).

Neurons in these areas integrate information coming from peripheral tissues, such as hormones and cytokines, nutrient metabolites, environmental cues, as well as neurohormonal and synaptic input from other areas of the brain regulate appetite and energy expenditure. Obesity develops through a disruption of this homeostasis, when animals are eating more than they are expending. With regard to appetite in particular, two distinct populations of cells located in the hypothalamic arcuate nucleus (ARC) are particularly relevant to energy balance regulation: the proopiomelanocortin cells (POMC) and the neuropeptide Y (NPY)/agouti-related protein (AgRP)-producing cells (Hahn, Breininger et al. 1998). Activation of POMC neurons leads to release of α-melanocyte stimulating hormone (α-MSH) which leads to activation of melanocortin receptor 4 (MC4R), leading to suppressed food intake and increased energy expenditure (Aponte, Atasoy et al. 2011). Conversely, activation of NPY/AgRP neurons leads to release of AgRP, which antagonizes the MC4R (Ollmann, Wilson et al. 1997; Bagnol, Lu et al. 1999), and also directly inhibits POMC perikarya (Tong, Ye et al. 2008; Wu, Howell et al. 2008). These ARC neurons respond directly to peripheral cues of energy balance, such as long term cues related to energy stores (Cowley, Smart et al. 2001; van den Top, Lee et al. 2004), and short term cues related to being in the fed or fasted state (Spanswick, Smith et al. 2000) (Andrews, Liu et al. 2008).

The ARC sends projections to the VMN, which contains a high density of receptors for both central and peripheral mediators of energy balance, but does not produce large amounts of orexigenic or anorexigenic peptides (Kalra, Dube et al. 1999). It is, however, involved in the control of autonomic responses that contribute to the prevention of obesity (reviewed by King (King 2006)). VMN lesions are associated with reduced sympathetic nervous system activity and delayed satiety leading to obesity (Vander Tuig, Knehans et al. 1982; Sakaguchi, Arase et al. 1988; Takahashi, Ishimaru et al. 1997). Activation of the VMN

elevates glycogenolysis (Takahashi, Ishimaru et al. 1997), promoting elevated serum glucose while attenuating appetite (Chen, Vaughan et al. 2010). Neurons of the VMN project to many areas associated with feeding behavior, including the amygdala (Saper, Swanson et al. 1976; Canteras, Simerly et al. 1994), ARC (Sternson, Shepherd et al. 2005), LH (Sclafani, Berner et al. 1975), PVN (Lin and York 2004), the DMN (Luiten and Room 1980; Ter Horst and Luiten 1987), as well as areas relating to rewarding aspects of feeding behavior including the VTA (Saper, Swanson et al. 1976), the NA, and the nucleus of the solitary tract (Canteras, Simerly et al. 1994). Recent evidence suggests the VMN contains large amounts of the vesicular glutamate transporter protein (Collin, Backberg et al. 2003; Meister 2007), suggesting that the VMN may modulate activity of these areas glutamatergic input to them. The VMN receives inputs from the Arc (Bagnol, Lu et al. 1999; Haskell-Luevano, Chen et al. 1999), the LH (Saper, Swanson et al. 1976; Ter Horst and Luiten 1987; Fahrbach, Morrell et al. 1989), and the amygdala (Luiten, Ono et al. 1983; Martinez-Marcos, Lanuza et al. 1999). Recent reports suggest that the fiber plexus lateral to the VMN contains axonal-dendritic synapses where dendrites from VMN neurons are in synaptic contact with axons likely from either PVN or SON, since the axons contain vesicular oxytocin (Griffin, Ferri-Kolwicz et al. 2010). These long primary dendrites extending from the VMN are shorter in food-restricted animals (Flanagan-Cato, Fluharty et al. 2008) suggesting they may have a role in the regulation of energy balance. Similarly, rats prone to diet-induced obesity have shorter long primary dendrites than those resistant to diet-induced obesity (Labelle, Cox et al. 2009). Whether these dendrites represent a connection from PVN or SON to the VMN is a compelling mystery with implications for revealing more of the picture of the hypothalamic regulation of energy balance.

The PVN contains magnocellular and parvocellular neurons involved in the initiation of stress responses (Bartanusz, Jezova et al. 1993; Givalois, Arancibia

et al. 2000). A population of corticotrophin-releasing hormone neurons exist in the parvocellular PVN (Sawchenko, Swanson et al. 1984). These neurons receive synaptic input from both orexigenic (NPY/AgRP) (Li, Chen et al. 2000) and anorexigenic (POMC/αMSH) neurons (Lu, Barsh et al. 2003) of the arcuate nucleus. αMSH secreted from POMC/αMSH neurons interacts with the MC4R in the PVN, and has potent anorexigenic effects (Balthasar, Dalgaard et al. 2005; Garza, Kim et al. 2008). The PVN contains high levels of brain derived neurotrophic factor (BDNF) and tropomyosin related kinase receptor B (trkB) mRNA (Tapia-Arancibia, Rage et al. 2004). In Chapter 3 I review BDNF in more thorough detail, and its role in the PVN related to energy balance.

Oxytocin

Oxytocin is a nine amino acid peptide hormone, neurohormone and neurotransmitter. The main sources of oxytocin in the brain are the magnocelluar and parvocellular neurons of the hypothalamic PVN and the supraoptic nuclei (Sokol, Zimmerman et al. 1976; Rosen, de Vries et al. 2008). Oxytocin is most known for its role in reproductive function including stimulating uterine contractions and lactation (Fuchs, Cubile et al. 1984; Fuchs, Rasmussen et al. 1984), but has also been reported to have a role in social bonding (Williams, Insel et al. 1994; Insel, Young et al. 1997), maternal behavior (Pedersen and Prange 1979) and anxiolytic responses (Altemus 1995; Heinrichs, Baumgartner et al. 2003). Increasingly, oxytocin is being recognized for its anti-obesity effects. Clinical trials are currently underway for its use in the treatment of obesity and type-2 diabetes (Ott, Finlayson et al. 2013; Zhang, Wu et al. 2013). Though the mechanism for oxytocin effects have not been fully characterized, an extensive body of work has demonstrated anti-obesity effects of oxytocin in rodents (Olson, Drutarosky et al. 1991; Deblon, Veyrat-Durebex et al. 2011; Zhang and Cai 2011; Morton, Thatcher et al. 2012; Zhang, Wu et al. 2013) as well as humans (Zhang,

Wu et al. 2013). Behaviorally, central oxytocin has been reported to reduce meal size (Lokrantz, Uvnas-Moberg et al. 1997; Yamashita, Takayanagi et al. 2013), delay meal onset (Arletti, Benelli et al. 1990) and reduce preference for sweet foods (Lokrantz, Uvnas-Moberg et al. 1997; Olszewski, Klockars et al. 2010). In addition to feeding effects, central oxytocin has potent effects on energy metabolism. For example, low doses of intracerebroventricular (i.c.v.) oxytocin promotes weight loss in rats without affecting feeding by elevating fat oxidation in adipose tissue, whereas higher doses of i.c.v. oxytocin both reduces feeding and increases lipolysis (Deblon, Veyrat-Durebex et al. 2011). In particular, PVN oxytocin production is essential to maintaining energy balance. This is illustrated by observations that SIM1 haploinsufficiency, which reduces PVN oxytocin expression by 80%, results in an obese hyperphagic phenotype, and is reversed by central oxytocin administration (Kublaoui, Gemelli et al. 2008). In the PVN, magnocellular neurons release oxytocin both somato-dendritically (Pow and Morris 1989) and via axon terminals, most of which project to the posterior pituitary (Swanson and Kuypers 1980) where oxytocin is released into peripheral circulation. Parvocellular oxytocin neurons send projections to median eminence, and additional central locations including spinal cord and brainstem (Swanson and Kuypers 1980; Rinaman 1998). Strong evidence exists implicating the nucleus of the solitary tract (NTS) as a site where oxytocin affects feeding and energy expenditure, however in this dissertation I report novel findings, that oxytocin in the VMN is a negative regulator of energy balance.

Overview of chapters

Herein I present a series of investigations intended to research mechanisms related to the hypothalamic regulation of energy balance. Of particular interest to me is the possibility that exercise might alter hypothalamic signaling in such a way as to promote the maintenance of a lower body weight via reduced feeding.

A detailed review of literature related to exercise affects on appetite is presented in Chapter 2. In this review, the possibility that exercise alters appetite via hypothalamic brain derived neurotrophic factor is touched upon. Chapter 3 is a more detailed review of the role of BDNF in the central regulation of energy balance. In Chapter 4 I present data from two behavioral experiments on the effects of exercise on feeding and energy balance and the role of BDNF in the hypothalamic paraventricular nucleus. Chapter 5 is a divergence from the topic of BDNF and exercise. In Chapter 5, I present data showing that oxytocin in the ventromedial hypothalamus reduces appetite and energy balance. The research and data presented herein spark many questions and highlight several promising avenues for discovery about how the brain regulates energy balance. This is the topic of Chapter 6, in which general conclusions and future directions are discussed.

Chapter 2

The fruits of fitness: paradoxical effects of exercise on the central regulation of appetite

Introduction

Rodent studies have long reported the interesting paradox of exercise-induced hypophagia (Edholm, Fletcher et al. 1955; Stevenson, Box et al. 1966). As early as 1970 it was proposed that the benefit of exercise in preventing obesity extends beyond increasing energy expenditure and includes the prevention of excessive feeding (Baile, Zinn et al. 1970). Despite metabolic demands of exercise, rodents will work to gain access to a running wheel (Iversen 1993), suggesting that they find the activity rewarding. In the context of a dietary challenge, where animals are presented with a palatable high fat diet (HFD), exercised rodents failed to increase their caloric intake (Krawczewski Carhuatanta, Demuro et al. 2011) or reduced feeding behavior (Shapiro, Cheng et al. 2011) compared with sedentary controls. In humans there is an inverse correlation between physical activity and body mass index among obese adults (Hemmingsson and Ekelund 2007) and an inverse association between energy expended in high intensity exercise and overweight/obesity (Tucker and Peterson 2003; Bernstein, Costanza et al. 2004). Similar to rodents, exercised humans have been reported to eat less than sedentary controls. In a randomized, controlled trial designed to simulate the metabolic effects of being in outer space, called the Women International Space Simulation for Exploration (WISE) study, those confined to complete bed-rest ate more than those on bed-rest who were on a moderate exercise program, despite the increased energy demands of the exercise (Bergouignan, Momken et al. 2010). Complete bed rest may be more

similar to housing conditions of singly housed laboratory rats than to intervention studies in free-living humans. As people become more sedentary, however, the effects of exercise on appetite and the mechanisms behind these effects compared with a sedentary lifestyle are increasingly relevant. Exercise normalizes insensitive appetite control in sedentary humans, as evidenced by reduced feeding at a buffet after a high-energy preload meal (Martins, Truby et al. 2007). Exercise increases the drive to eat during fasting, but it also improves satiety, which was determined by a satiety quotient calculated from appetite scores after consumption of a fixed quantity meal (King, Caudwell et al. 2009; Martins, Kulseng et al. 2010).

Much of the rodent data reviewed herein is from male rats, unless otherwise specified. Recent reports have addressed the issue that the data are substantially less abundant with regards to how female rats respond to exercise (Schroeder, Shbiro et al. 2010). There are some reports in which exercise did not alter, or even increased feeding, however in these cases rats usually failed to increase their caloric intake to compensate for energy expended. Female rats, however, appear to respond differently to exercise than males. The type of rat model, and more specifically responsiveness to insulin and leptin may influence sensitivity to the anorexigenic effects of exercise. Potential mechanisms of the anorexigenic effects of exercise are reviewed herein, and likely involve hypothalamic sensing of nutrients, hormones, growth factors and cytokines.

Rodent models

Obesity prone and rats with diet-induced obesity

Rats selectively bred to be prone to diet-induced obesity (OP) have been useful in investigations about how exercise affects the development of obesity.

Access to running wheels (RW) in juvenile rats four weeks post-weaning is associated with reduced adiposity and failure to increase feeding of a palatable HFD, despite the increased energy cost of running (Patterson, Dunn-Meynell et al. 2008). Remarkably, OP exercised animals refrained from overeating the HFD even after RW were locked, while food restricted animals that did not exercise but whose body weight and food intake were similar to animals that ran, ate significantly more HFD on ad libitum feeding (Patterson, Dunn-Meynell et al. 2008). Thus, while both food restriction and exercise promoted similar protection against HFD-induced weight gain, exercise benefits extended beyond the intervention time to protect against relapse weight gain, whereas food restriction without exercise did not. Even after the wheels had been locked for seven weeks, OP animals that had previously been exercised remained leaner than OP animals never exposed to a wheel, suggesting that exercise during a critical period of development normalizes homeostatic mechanisms and prevents obesity in those susceptible (Patterson, Dunn-Meynell et al. 2008).

Others have reported that 12-hour food intake is reduced in diet induced obese (DIO) animals after a single bout of either swimming or treadmill running, however exercise had no effect on feeding in lean animals compared with sedentary controls (Ropelle, Flores et al. 2010). Similarly, lean rats fed standard chow that were exercised for 120 min/day on a treadmill lost weight but had a similar food intake to sedentary controls (Jenkins and Lamb 1982). Thus while lean animals may not reduce feeding in response to exercise, they also fail to increase feeding to compensate for calories expended. Others have reported that rats fed standard chow significantly reduced their food intake during the first 37 days of running, but after the initial reduction these animals increased their food intake such that when the study was terminated there were no differences in cumulative food intake between groups (Shapiro, Matheny et al. 2008). While exercise reduces feeding in the context of obesity, there may be protective

mechanisms, which prevent animals from becoming too lean. The effect of exercise on feeding is less robust in lean animals that have never been obese, than in those that have been previously obese. In DIO rats calorically restricted to 14% weight reduction, treadmill exercise reduced the drive to overeat when allowed to eat ad libitum, conferring maintenance of lower body weight (MacLean, Higgins et al. 2009), particularly in the initial stages of weight re-gain (Higgins, Jackman et al. 2011). The discrepancy between feeding response to exercise in lean and obese rats, and in those either naïve to obesity or previously DIO, suggests that sensitivity to signals related to energy balance might play a role in affecting anorexigenic responses to exercise. In support of this idea, rodents with RW access raised on HFD ran a negligible amount, and after 5 weeks exercise or being sedentary, sedentary rats fed HFD had similar food consumption to animals with RWs, but when leptin was overexpressed in HFD-fed animals, exercising animals ate 20% less than sedentary animals with leptin overexpression (Shapiro, Matheny et al. 2008). Similarly, after an acute bout of endurance swimming, there was a slight non-significant food reduction in exercised animals compared with controls, however leptin or insulin increased anorexigenic effects of exercise in a dose-dependent manner (Flores, Fernandes et al. 2006). Taken together, these data suggest that exercise may have some beneficial effect on appetite during juvenile development, and that the anorexigenic effects of exercise may be particularly robust in animals prone to DIO or previously DIO, and are enhanced with leptin and insulin.

The Otsuka Long-Evans Tokushima fatty rat

The Otsuka Long-Evans Tokushima fatty (OLETF) rat is an obesity model in which animals become hyperphagic due to lack of a functional cholecystokinin receptor 1 (CCK-1R). Cholecystokinin (CCK) is a hormone that acts centrally to

reduce food intake (Blevins, Stanley et al. 2000; Reidelberger, Hernandez et al. 2004). As a consequence of lacking the CCK-1R, OLETF rats have a pattern of eating larger meals (Moran, Katz et al. 1998) and they develop obesity secondary to hyperphagia (Bi, Ladenheim et al. 2001; Moran and Bi 2006). Exercise prevents obesity in OLETF rats (Shima, Shi et al. 1993) primarily by reducing meal size (Bi, Scott et al. 2005). After six weeks of RW access beginning at eight weeks old, OLETF rats were denied access to wheels for a subsequent six weeks, during which time feeding, body fat and leptin increased compared with exercising rats, but remained lower than sedentary controls (Bi, Scott et al. 2005). Similarly, feeding and body fat remained lower in OLETF rats given four weeks of RW access early in life, even after the wheels had been locked for eight weeks (Chao, Terrillion et al. 2011). These data are in agreement with what has been described in OP rat models: that there appears to be a critical phase of development where exercise promotes obesity resistance for prolonged time periods, such as for seven weeks post exercise cessation (Patterson, Dunn-Meynell et al. 2008). Interestingly, preventative effects of early exercise on obesity in OLETF rats fed standard chow were overwhelmed by HFD (Chao, Terrillion et al. 2011). CCK is generally secreted during consumption of HFD, and therefore OLETF rats are particularly vulnerable to HFD-induced obesity (Bi, Chen et al. 2007). It is likely that the anorexigenic effects of exercise reported previously in DIO rats (Patterson, Dunn-Meynell et al. 2008; Patterson, Bouret et al. 2009; Ropelle, Flores et al. 2010) (MacLean, Higgins et al. 2009; Higgins, Jackman et al. 2011) require functional CCK-1R signaling. It is also possible, as Chao et al speculate (Chao, Terrillion et al. 2011), that the composition of fat in the diet (60% for Chao et al) or the timing of the introduction of HFD relative to the introduction of the wheel may explain the observed discrepancy in feeding response. Shapiro et al observed that rats made obese by previous exposure to HFD significantly reduced their food intake when given access to RW (Shapiro,

Cheng et al. 2011). Similar to Chao et al, these rats were fed 60% kcal from fat; however, Shapiro et al used F344 x Norway Brown rats, which have functional CCK-1Rs, supporting the idea that anorexigenic effects of exercise may require functional CCK-1R. Thus dietary composition is likely not the sole factor for driving the discrepancy between feeding responses, but the timing of the wheel introduction or CCK-1R discrepancy may play a role.

Shapiro et al also observed that access to RW reduced feeding even when animals ran a small amount (as little as 9 revolutions per day), and that leptin signaling was enhanced in the VTA, but not hypothalamus, suggesting a possible hedonic substitution of the wheel for food (Shapiro, Cheng et al. 2011). Leptin and CCK act synergistically to promote satiety (Emond, Schwartz et al. 1999; Peters, Simasko et al. 2006) specifically via the CCK-1R (Barrachina, Martinez et al. 1997) and leptin receptors (Heldsinger, Lu et al. 2012). Vagal afferents from the nodose ganglia (NG) are sites of leptin and CCK action, which mediate short-term satiety signals via cocaine and amphetamine regulated transcript (Heldsinger, Lu et al. 2012). VTA leptin has been reported to enhance the satiety promoting effects of peripheral CCK (Morton 2007). Thus, in HFD-feeding paradigms, exercise may reduce feeding by increasing VTA leptin, which would enhance the satiety promoting effects of CCK.

During the post-weaning period, both OLETF male rats and male Long-Evans Tokushima (LETO) controls reduce feeding in response to exercise, and in the case of male OLETF rats, there was sustained reduction in feeding and body weight after the running wheels were locked (Schroeder, Shbiro et al. 2010). This is similar to reports on OP rats regarding early prevention of obesity with exercise, where early access to running promotes sustained protection against weight gain even after exercise is removed (Patterson, Dunn-Meynell et al. 2008; Patterson, Bouret et al. 2009). Interestingly, female OLETF rats had a moderate feeding reduction in response to exercise; however unlike males, exercised

female LETO rats increased caloric intake, indicating a sexually dimorphic effect (Schroeder, Shbiro et al. 2010). Exercised female OLETF rats had increased brown fat and reduced feeding efficiency even without differences in white adipose tissue between exercised and sedentary groups (Schroeder, Shbiro et al. 2010). In summary, male OLETF rats on standard chow have an anorexigenic and obesity preventative response to RW that may be prolonged for several weeks once RWs are no longer available, this effect was less pronounced in female OLETF rats. HFD reduces the anorexigenic effects of exercise in OLETF rats, which may be due to the importance of CCK as satiety signal during HF feeding.

Zucker fatty (fa/fa) rat

The Zucker fatty rat has impaired functioning of the leptin receptor due to a missense gene mutation (Phillips, Liu et al. 1996; Takaya, Ogawa et al. 1996; Crouse, Elliott et al. 1998). The fa/fa rat is often used as a model for type 2 diabetes, as these rats are hyperinsulinemic and insulin resistant. In this model, some have reported that treadmill exercise has minimal or no effect on feeding or body weight gain in animals fed standard chow (Santti, Huupponen et al. 1994; Colombo, Gregersen et al. 2005). Exercise alone did not affect feeding in Zucker fa/fa rats, however, again in this model rats failed to increase caloric intake to compensate for increased energy demands (Santti, Huupponen et al. 1994). Conversely, others have reported that moderate swimming exercise reduced feeding in fa/fa rats fed standard chow, but this effect was absent when animals were offered a palatable HFD (Kibenge and Chan 2002). BRL-35135 is a β-adrenergic receptor agonist that selectively targets brown adipose tissue, and stimulates thermogenesis, increases metabolic rate, and improves glucose tolerance (Cawthorne, Sennitt et al. 1992). The combination of physical exercise

and oral BRL-35135 reduced food intake in male fa/fa rats where neither alone was sufficient to do so (Santti, Huupponen et al. 1994). In female lean Zucker rats, intense exercise training (2 hours/day at 20m/min) increased feeding and body weight compared with sedentary rats, while obese Zucker fatty fa/fa exercise trained rats were lighter and had similar food intake to sedentary controls (Wardzala, Crettaz et al. 1982). The increased feeding and body weight reported in female, lean Zucker rats is similar to what has been reported elsewhere for females (Titchenal 1988; Scheurink, Ammar et al. 1999; Schroeder, Shbiro et al. 2010; Carrera, Cerrato et al. 2011). In female Zucker rats, exercise is associated with increased utilization of glucose by adipocytes and increased sensitivity to epinephrine, reflecting an enhanced capacity for triglyceride turnover (Wardzala, Crettaz et al. 1982). Taken together, exercise had anorexigenic effects in males, but this effect was absent with HFD-feeding. Lean female Zucker rats consumed more food to compensate for training deficits despite enhanced capacity for fat utilization, whereas obese females lost weight with exercise. The reported gender dimorphism may represent a biological adaptation where females preserve body fat for reproductive purposes.

Potential mechanisms

Specific areas of the hypothalamus have been extensively shown to be involved in the regulation of feeding behavior (Steffens, Scheurink et al. 1988). Using the immediate early gene transcription factor c-Fos as a marker of neuronal activity, several hypothalamic areas were activated 1.5 hours post acute treadmill running at above lactate threshold (25 m/min), including the medial preoptic area (MPO), periventricular nucleus (Pe), supraoptic nucleus (SON), parvocellular paraventricular nucleus (pPVN), the anterior hypothalamic area (AH), the arcuate nucleus (Arc) and the posterior hypothalamus (PH) (Soya,

Mukai et al. 2007). Though the ventromedial hypothalamus (VMN), dorsomedial hypothalamus (DMH) and lateral hypothalamus (LH) have all been shown to affect food intake, none of these areas were activated, as indicated by elevated c-Fos, after an acute bout of intense treadmill exercise (Soya, Mukai et al. 2007). In contrast, Krawczewski et al report increased FosB immunoreactivity in the dorsomedial VMN of mice after 4 weeks of exercise, with no changes in FosB in other areas including PVN and Arc (Krawczewski Carhuatanta, Demuro et al. 2011). The data presented by Krawczewski et al reflect that exercise did not reduce feeding in C57B6J mice compared with sedentary animals, however these mice did fail to increase their caloric intake to compensate for increased energy demands, causing a negative energy balance (Krawczewski Carhuatanta, Demuro et al. 2011).

Neurons of the hypothalamus, particularly the ARC, express orexigenic NPY and anorexigenic POMC. Exercise has been reported to increase hypothalamic NPY (Lewis, Shellard et al. 1993), however this is controversial and may be confounded due an initial period of food restriction which was used to motivate the animals to exercise. Nevertheless, others have reported that, in cases where animals were not food restricted, exercise increases Arc NPY expression in both Long-Evans Tokushima rats and OLETF rats, despite reduced feeding behavior (Bi, Scott et al. 2005). Conversely, light intensity (5 m/mn for 30 min) but not moderate or high intensity treadmill exercise reduced NPY expression in the Arc and PVN of rats with STZ-induced diabetes (Shin, Kim et al. 2003). Similarly, it has been reported that exercise normalizes the increased fasting NPY and reduced POMC associated with chronic food excess (Ropelle, Flores et al. 2010). Exercising OP rats that had previously been weight restricted, but were then allowed to eat ad libitum maintained a lower body weight than sedentary controls. There were no differences in hypothalamic NPY expression in the ARC or DMN, however POMC was lower in the Arc of exercising rats

(Levin and Dunn-Meynell 2004). Conversely, in OP rats with RW fed HFD, exercise was associated with higher POMC and higher DMN NPY (Patterson, Dunn-Meynell et al. 2008). In DIO rats, hypothalamic NPY is increased and ghrelin reduced for the first 3 hours post exercise, however by 12 and 24 hours post exercise ghrelin was elevated and NPY normalized to baseline levels (Wang, Chen et al. 2008). Thus the effect of exercise on hypothalamic NPY and POMC is inconsistent across the literature.

Alpha-melanocyte stimulating hormone (αMSH) secreted from POMC/αMSH neurons interacts with the melanocortin 4 receptor (MC4R) in the PVN, and has potent anorexigenic effects (Balthasar, Dalgaard et al. 2005; Garza, Kim et al. 2008). The Ay mouse has ectopic expression of agouti, which antagonizes the binding of αMSH to the MC4R resulting in obesity and hyperphagia (Leibel, Chung et al. 1997; Miltenberger, Mynatt et al. 1997). Exercise slows weight gain in Ay mice (Goodrick 1978; Chiu, Fisler et al. 2004) and reduces food intake to the level of exercising controls (Chiu, Fisler et al. 2004). These data suggest that hypothalamic αMSH signaling is not required for exercise effects on energy balance.

Leptin and insulin

Krawczewski et al reported that the FosB expression colocalizes with leptin receptor in cells in the VMN, and consequently exercised animals were more sensitive to anorexigenic effects of an ICV leptin injection (Krawczewski Carhuatanta, Demuro et al. 2011). Plasma levels of the adipokine leptin are lowered in proportion to body fat reductions consequent to exercise, however several studies have shown that exercise enhances brain sensitivity to leptin (Flores, Fernandes et al. 2006; Patterson, Bouret et al. 2009; Ropelle, Flores et al. 2010; Krawczewski Carhuatanta, Demuro et al. 2011), or that exercise and

leptin act synergistically to reduce feeding (Shapiro, Matheny et al. 2008). Leptin may cross the blood-brain barrier via a putative leptin transporter, which is present in the median eminence and Arc (Banks, Kastin et al. 1996). Kimura et al reported reduced mRNA for hypothalamic leptin receptor (ObRb) after twelve weeks of RW (Kimura, Tateishi et al. 2004), however others have described increases in leptin receptor protein in whole hypothalamus following exercise (Gomez-Pinilla and Ying 2010). Patterson et al. observed a persistent reduction in diet-induced obesity in juvenile rats selected for sensitivity to DIO after three weeks of exercise training during the post-weaning period (Patterson, Dunn-Meynell et al. 2008; Patterson, Bouret et al. 2009). Similarly, Bi et al observed reduced BW in OLETF rats given access to RW during post-weaning period, which persisted after the wheels were locked. The resistance to weight gain persisted for ten weeks after the cessation of exercise, and was associated with increased sensitivity to both thermogenic and anorectic effects of leptin (Patterson, Bouret et al. 2009). The ability of exercise to enhance leptin signaling is not limited to the juvenile rat brain. Ten week old HFD-fed mice have a low anorectic response to leptin injections, however HFD-fed exercised mice demonstrate increased sensitivity to ICV leptin, as indicated by reduced feeding and body weight (Krawczewski Carhuatanta, Demuro et al. 2011). Leptin sensitivity is also increased with exercise in rats fed standard chow. In support of this, a single bout of prolonged exercise was sufficient to enhance hypothalamic leptin sensitivity, as measured by reduced feeding and activation of leptin receptor signaling pathways (Flores, Fernandes et al. 2006). Additionally, lean rats fed standard chow failed to increase their food intake to compensate for caloric demands of nine weeks of chronic exposure to endurance treadmill exercise (up to one hour per day) (Zhao, Tian et al. 2011). These rats had a 60% increase in leptin receptor mRNA as well as increased hypothalamic activation of several signaling factors indicative of leptin receptor activation, including Janus

kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), suppressor of cytokine signaling 3 (SOCS3), as well as protein kinase B (Akt) and extracellular related kinases (ERKs) (Zhao, Tian et al. 2011). Others have reported that RW exercise increases activation of SOCS3 and STAT3 in leptin overexpressing rats (Shapiro, Matheny et al. 2008). The extent to which endogenous activation of the leptin receptor contributes to increased satiety with exercise is not known. However, Ropelle et al. reported that a single bout of either swimming or treadmill exercise reduced the food intake of leptin deficient ob/ob mice but not wild type controls (Ropelle, Flores et al. 2010), suggesting a leptin independent mechanism for exercise mediated reductions in food intake. Thus reduced feeding associated with exercise is likely partially or minimally due to increased leptin sensitivity, if at all. Leptin reduces food intake by increasing POMC neuronal activity and decreasing NPY/Agrp in the hypothalamus, but also regulates food intake via extra-hypothalamic sites (for review see Morris and Rui 2009 (Morris and Rui 2009)). In the hypothalamus, the anorectic effects of leptin are thought to be largely mediated via αMSH, which comes from the precursor POMC, however RW exercise was associated with reduced feeding in MC4R knock out mice (Irani, Xiang et al. 2005). Conversely, others have shown that exercise is associated with increased feeding in MC4R knockout mice, however overall these animals were less calorically efficient and thus more resistant to obesity than sedentary MC4RKO animals (Haskell-Luevano, Schaub et al. 2009). This, taken together with the conflicting reports of NPY expression in exercising animals, indicates that exercise anorexigenic effects do not require leptin to act via increased POMC or reduced hypothalamic NPY expression, and may not require leptin signaling.

Exercise increases glycogenolysis and results in depleted glycogen stores. Recent evidence suggests that brain glycogen, including hypothalamic glycogen, is depleted during exhaustive exercise resulting in a period of glycogen

supercompensation (Matsui, Ishikawa et al. 2012). Brain glycogen supercompensation is similar to muscle supercompensation, but precedes it (Matsui, Ishikawa et al. 2012). Thus the body replenishes glucose supplies in the brain first before replacing what is lost from the periphery. This is in line with the "selfish brain theory", which suggests that both leptin (which activates the sympathetic nervous system) and insulin convey signals that result in increased glucose to the brain, and thus reduce appetite (Peters, Schweiger et al. 2004). As peripheral glycogen stores are depleted, the muscles become temporarily insulin resistant (Kirwan and Jing 2002). However, exercise improves intrahypothalamic insulin sensitivity in animals made less insulin responsive by chronic overnutrition (Ropelle, Flores et al. 2010). This effect is accompanied by increased activation of insulin receptor, IRS-1, and IRS-2 (Flores, Fernandes et al. 2006). In order to coordinate central and peripheral insulin sensitivity with glycogen storage status, the peripheral and central nervous system communicate potentially via cytokines (for review see (Steinacker, Lormes et al. 2004)). In particular interleukin-6 (IL-6) is a good candidate, because it is released during exercise in amounts proportional to pre-exercise muscle glycogen content (Steensberg, van Hall et al. 2000; Steinacker, Lormes et al. 2004). Thus fluctuations in glucose may affect feeding behavior via direct sensing, indirectly via insulin receptor signaling or through other cytokines.

Interleukin-6

Interleukin-6 (IL-6) is a cytokine which signals by forming a complex with the cytokine selective co-receptor IL-6 receptor and the transmembrane protein glycoprotein 130 (gp130) (Taga, Hibi et al. 1989; Hibi, Murakami et al. 1990; Murakami, Hibi et al. 1993). IL-6 deficient (knockout) mice develop obesity, which can be reversed by giving ICV IL-6 injections (Wallenius, Wallenius et al. 2002).

In Wistar rats, ICV IL-6 injections reduced body fat, leptin, and total food intake (Wallenius, Wallenius et al. 2002). The leptin receptor is a member of the class 1 (or hemopoietin) cytokine receptor family and acts independently of gp130 (Tartaglia, Dembski et al. 1995; Wang, Kuropatwinski et al. 1997). Both leptin and IL-6 are activators of STAT3, and thus have similar downstream intracellular effects (Wang, Kuropatwinski et al. 1997). IL-6 may be a key player in mediating exercise effects on appetite and energy balance through central nervous system signaling (Flores, Fernandes et al. 2006; Ropelle, Flores et al. 2010; Zhao, Tian et al. 2011). Like leptin, circulating IL-6 is produced by adipose tissue (Keller, Keller et al. 2003) however, unlike leptin circulating IL-6 also originates from working skeletal muscle (Keller, Keller et al. 2003; Penkowa, Keller et al. 2003) and is increased by exercise (Steensberg, van Hall et al. 2000). Additionally, IL-6 is greatly expressed in anorexigenic and orexigenic neurons of the Arc (Ropelle, Flores et al. 2010), and the IL-6 receptor is widely expressed in the Arc (Ropelle, Pauli et al. 2008). When injected into the third ventricle, IL-6 dose dependently reduces food intake in obese animals, and infusions of an anti-IL-6 antibody inhibits exercise-induced improvements on the anorexigenic effects of insulin and leptin (Flores, Fernandes et al. 2006; Ropelle, Flores et al. 2010). Exercise and IL-6 increase the phosphorylation of hypothalamic Akt (a marker of insulin receptor activation) and STAT3 in DIO animals. This effect is blocked in exercised animals by pretreatment with an IL-6 antibody (Flores, Fernandes et al. 2006; Ropelle, Flores et al. 2010). The extent to which endogenous IL-6 contributes to exercise effects on energy balance is controversial. It has been reported that up to 2 hours a day of treadmill running did not have a significant effect on either serum or hypothalamic levels of IL-6 compared with sedentary controls, despite the reduced weight gain in treadmill exercised animals (Chennaoui, Drogou et al. 2008). Thus, further investigation is needed to

determine how much endogenous IL-6 signaling contributes to exercise effects on energy balance.

AMP-activated protein kinase

AMP-activated protein kinase (AMPK) is a signaling molecule found in both central and peripheral tissue. Through its role as a member of a protein kinase cascade, central AMPK can directly affect feeding behavior. AMPK activity is modulated by cytokines and hormones involved in energy balance. For example, leptin inhibits central AMPK, particularly in the ARC and PVN (Minokoshi, Alquier et al. 2004), whereas plasma ghrelin increases hypothalamic AMPK activity and stimulates feeding (Andersson, Filipsson et al. 2004). Intracellularly, AMPK is activated by low energy states, specifically by a high ratio of AMP:ATP, consequently activation of central AMPK leads to increased food intake, whereas inhibition leads to reduced feeding (Andersson, Filipsson et al. 2004; Kim, Park et al. 2004; Minokoshi, Alquier et al. 2004). Acute bouts of treadmill running lasting either 30 minutes or 1 hour had no effect on AMPK activity, however, food intake was not reported in this study and it is possible that exercise did not alter feeding in this case (Andersson, Treebak et al. 2005). Ropelle et al reported that, though prolonged endurance exercise (3 hours of swimming, followed by 45 minutes of rest and an additional 3 hours of swimming) alone had no effect on feeding in young male Wistar rats, exercise enhanced the anorexigenic effects of leptin, which were associated with reduced AMPK and the downstream acetyl-coA carboxylase (ACC) phosphorylation (Ropelle, Pauli et al. 2008). This was true in both normal weight and DIO rats (Ropelle, Pauli et al. 2008). It is not uncommon for DIO rodents, and potentially those susceptible to DIO, to be resistant to or less sensitive to leptin (Prpic, Watson et al. 2003; Munzberg, Flier et al. 2004; Flores, Fernandes et al. 2006; Martin, Alquier et al.

2006). Thus it is possible that anorexigenic effects of exercise are most prominent in cases where animals are less sensitive to leptin. Exercise promoted greater reductions in feeding after ICV infusion of either leptin or α-linoleic acid (which suppresses AMPK activity (Kim, Park et al. 2004)), and blunted increased feeding in response to AMPK activators AICAR, 2-DG or fasting (Ropelle, Pauli et al. 2008). Interestingly, Andersson et al observed a 40% ghrelin increase following a 1-hour run, yet AMPK activity was not increased (Andersson, Treebak et al. 2005). Using both AICAR and 2DG, both pharmacological activators of AMPK, Ropelle et al reported that exercise interferes with AMPK activation (Ropelle, Pauli et al. 2008), thus it is tempting to consider that exercise may also interfere with the ghrelin-induced activation of AMPK. Though some studies have reported no effect of exercise on hypothalamic IL-6 (Chennaoui, Drogou et al. 2008), one study reported that hypothalamic IL-6 was increased by 420% in exercised rats, resulting in decreased AMP: ATP and consequently reduced AMPK phosphorylation (Ropelle, Pauli et al. 2008). In the case where hypothalamic IL-6 was increased, pretreatment with an IL-6 antibody blunted exercise-induced anorexigenic effects of leptin, supporting an essential role for IL-6 in exercise-mediated anorexigenic effects of leptin (Ropelle, Pauli et al. 2008). Thus it is possible that exercise enhances leptin signaling via an AMPK dependent pathway, which requires functional use of the IL-6 receptor. In summary, exercise has been reported, in some cases, to interfere with hypothalamic AMPK activation. This may be a direct consequence of nutrient utilization (a high AMP: ATP ratio), or may be related to exercise effects on sensitivity to peripheral hormones such as leptin and ghrelin.

Nutrient sensing

The hypothalamus contains neurons sensitive to both free fatty acids (Oomura, Nakamura et al. 1975; Lam, Pocai et al. 2005; Migrenne, Marsollier et al. 2006; Le Foll, Irani et al. 2009) and glucose (Wang, Liu et al. 2004) and, therefore, to metabolic status. Glucose can act as a signaling molecule to the brain to affect food intake, as evidenced by early experiments whereby administration of 2 deoxyglucose (2DG) into the third ventricle resulted in hyperphagia (Miselis and Epstein 1975). A few areas of the hypothalamus are most prominent in the study of glucosensing neurons, namely the VMN (Ono, Nishino et al. 1982), LH (Oomura, Kimura et al. 1964; Oomura, Ono et al. 1969), and Arc (Wang, Liu et al. 2004). Central administration of glucose inhibits food intake, however, since glucose is the preferred fuel for the brain, generally brain glucose is maintained within a tight range (0.5-2.5mM) (Watts and Donovan 2010). Nevertheless, reductions in plasma glucose stimulate feeding (Campfield and Smith 2003). As previously discussed, exercise increases sensitivity to leptin and insulin, but exercise also affects substrate utilization. Exercise trained animals had increased dietary fat oxidation and de novo lipogenesis during the first 24 hours of overfeeding after weight restriction, indicating the preferential oxidation of fat and storage of carbohydrate and protein, which is more energetically costly (Steig, Jackman et al. 2011). These differences in fuel utilization may lead to reduced appetite due to nutritional signals feeding back to the brain from the periphery (Steig, Jackman et al. 2011). Aerobic exercise increases non-esterified free fatty acids (NEFA) in obese (Ropelle, Flores et al. 2010) and weight-reduced rats fed ad-libitum after a previously bout of caloric restriction (Steig, Jackman et al. 2011). This is similar in humans, as exercise increases post-prandial NEFA (Kokalas, Petridou et al. 2005). Circulating NEFA reduces food intake (Vandermeerschen-Doize and Paquay 1984), as does central administration of long chain FFA (Obici, Feng et al. 2002; Morgan, Obici

et al. 2004; Schwinkendorf, Tsatsos et al. 2011). In the hypothalamus, the anorexigenic effects of oleic acid were mediated via MC4R signaling (Schwinkendorf, Tsatsos et al. 2011). Interestingly, reduced plasma FFA activates hypothalamic arginine vasopressin, which causes increased ACTH and corticosterone release (Oh, Oh et al. 2012). Thus the hypothalamus senses when plasma FFA are low and responds by activating the hypothalamic pituitary adrenal axis, the end result of which is an increase in orexigenic glucocorticoids (Oh, Oh et al. 2012). On the other hand, others have previously reported that ICV infusions of NEFA are associated with increased glucocorticoids, circulating insulin and hepatic glucose production (Clement, Poirier et al. 2002). Reasons for the discrepancy are likely due to location of the FFA infusion (IV vs ICV). Subsequent studies utilized intracarotid infusion techniques to bathe the brain in FFA but not bypass the blood brain barrier (Migrenne, Marsollier et al. 2006). In this paradigm, plasma FFA were capable of stimulating plasma insulin without affecting plasma glucose (Migrenne, Marsollier et al. 2006), thus central NEFA can affect hormones related to feeding. Similarly, intravenous infusions of NEFA reduce circulating ghrelin (Gormsen, Gjedsted et al. 2006), plasma corticosterone and ACTH (Oh, Oh et al. 2012). It has been reported that a prolonged period of exercise training (8 weeks) reduces NEFA compared with sedentary DIO rats (Gauthier, Couturier et al. 2003; Chapados, Collin et al. 2008). In these cases, samples were obtained from fasted rats. Thus it is possible that post-prandial NEFA may contribute to the anorexigenic effects of exercise in trained DIO, or DIO susceptible rats.

Hormonal signals from the enteric nervous system relay information to the brain about the presence or absence of nutrients; for example, ghrelin and peptide Y (PYY) interact with leptin and insulin in the CNS and regulate energy balance. Ghrelin is orexigenic and promotes the release of NPY and Agrp from neurons of the hypothalamic Arc (Wren, Small et al. 2000; Kamegai, Tamura et

al. 2001; Wren, Seal et al. 2001; Wren, Small et al. 2001). PYY is released from the gastrointestinal tract during feeding in proportion to the caloric content of the meal (Batterham, Cowley et al. 2002). Wang et al. recently reported the reduced feeding and body weight gain in obese rodents after long term exercise were associated with reduced hypothalamic ghrelin (Wang, Chen et al. 2008), but others have reported that plasma concentrations of ghrelin are increased during acute bouts of exercise (Andersson, Treebak et al. 2005). Plasma PYY does not change with exercise and has been reported to decrease during post exercise re-feeding (Andersson, Treebak et al. 2005).

Inflammation

It has been proposed that the development of obesity involves the disregulation of central leptin and insulin signaling by proinflammatory molecules, which are activated by overnutrition (De Souza, Araujo et al. 2005; Zhang, Zhang et al. 2008; Milanski, Degasperi et al. 2009). In a recent study Yi et al. reported that mice with ad libitum access to a “western” diet developed hypothalamic inflammation, which moderate-intensity treadmill exercise alleviates (Yi, Al-Massadi et al. 2012). However, despite the long duration of the study (26 weeks) and the dramatic differences in ARC microglial activation between the two groups, there were no differences in food intake, leptin, body weight, or energy expenditure between exercised and sedentary animals (Yi, Al-Massadi et al. 2012). It is likely that the intensity of the training was too low to affect food intake (5 m/min for 30 min/day) and the authors were unable to detect differences in circulating pro-inflammatory cytokines between the two groups (Yi, Al-Massadi et al. 2012). Thus future studies are needed to determine either whether reductions in activated microglia contribute to obesity prevention or whether intense exercise sufficient to reduce food intake affects hypothalamic integrity during overfeeding.

Cancer anorexia/cachexia is associated with elevated hypothalamic pro-inflammatory cytokines interleukin-1 (IL-1Beta) and tumor necrosis factor alpha (TNF-alpha). In cancer anorexia/cachexia, while endurance exercise normalized hypothalamic protein levels of IL-1Beta and TNF-alpha after 8 weeks, food intake was not increased (Lira, Yamashita et al. 2011). This suggests that factors other than pro-inflammatory IL-1Beta and TNF-alpha modulate reduced feeding associated with cancer anorexia/cachexia.

HPA axis activation

Exercise activates the sympathetic nervous system and hypothalamic pituitary adrenal (HPA) axis (Girard and Garland 2002; Droste, Gesing et al. 2003; Droste, Chandramohan et al. 2007), resulting in increased epinephrine and glucocorticoid production, respectively. Chronic activation of the HPA axis can result in glucocorticoid excess, which is associated with central adiposity (Rosmond, Dallman et al. 1998), insulin resistance, hyperlipidemia, and increased glucose production (Saruta, Suzuki et al. 1986). Exercise, however, is associated with improved blood glucose regulation, reduced central adiposity (Giannopoulou, Fernhall et al. 2005; Giannopoulou, Ploutz-Snyder et al. 2005), and reduced insulin resistance (Richter, Garetto et al. 1982). It has been reported that HPA axis activity adapts after several weeks of exercise, which may explain why hyperglucocorticoidemia is not observed with chronic exercise (Fediuc, Campbell et al. 2006). Forced treadmill (Timofeeva, Huang et al. 2003), swimming (Jiang, Kawashima et al. 2004) and voluntary wheel exercise (Cao, Choi et al. 2011) increase CRH in the PVN and DMH (Bi, Scott et al. 2005) of rodents,, but these increases do not always result in glucocorticoid excess (Campbell, Kiraly et al. 2010), as many studies report no differences in circulating glucocorticoids when comparing exercised and sedentary animals, despite

elevated ACTH (Jankord, Ganjam et al. 2008; Campbell, Kiraly et al. 2010). Additionally, exercise training promotes habituation (reduced glucocorticoid responses) to certain types of stressors in rodents, such as moderate noise, i.p. saline injection, or exposure to novel environments (Droste, Chandramohan et al. 2007; Sasse, Greenwood et al. 2008; Campeau, Nyhuis et al. 2010), but exercise training either increased or had no effect on glucocorticoid responses to potentially physiologically demanding stressors (e.g. forced swimming (Droste, Chandramohan et al. 2007), predator odor, or restraint stress (Campeau, Nyhuis et al. 2010)). Thus exercise might prevent stress related weight gain by reducing glucocorticoid responses to stressors that do not require energy expenditure.

The HPA axis is initiated by corticotropin releasing hormone (CRH) produced by neurons of the paraventricular hypothalamus (PVN) (Sawchenko, Swanson et al. 1984). CRH neurons receive synaptic input from both orexigenic (NPY/AgRP) (Li, Chen et al. 2000) and anorexigenic (POMC/αMSH) neurons (Lu, Barsh et al. 2003) of the arcuate nucleus. PVN MC4R anorexigenic effects on food intake require CRH receptor signaling (Lu, Barsh et al. 2003). Adrenalectomized animals have increased PVN CRH expression due to lack of negative feedback from glucocorticoids, which results in anorexigenic effects that are abolished by icv pretreatment with the CRH R2 antagonist (Uchoa, da Silva et al. 2010). Recent evidence suggests that exercise is associated with reduced adrenal ACTH receptor, and thus chronic exercise may reduce sensitivity to ACTH at the adrenal level (Campbell, Kiraly et al. 2010). In addition to being an initiator of the HPA axis, CRH acts on CRH receptors in the brain to reduce feeding (Krahn, Gosnell et al. 1986; Arase, York et al. 1988; Uchoa, Sabino et al. 2009). CRH both reduces energy intake and increases energy expenditure (Richard, Lin et al. 2002) acting centrally through the CRH receptor 1 (CRH R1) and 2 (CRH R2) (Perrin, Donaldson et al. 1993; Lovenberg, Liaw et al. 1995). Kawaguchi et al observed that central ICV administration of a CRH R1/R2

receptor antagonist, alpha helical CRH, abolished the anorectic effects of running, mainly by preventing exercise induced reductions in meal size (Kawaguchi, Scott et al. 2005). While Kawaguchi et al did not observe increases in PVN CRH expression with exercise, they did observe dramatic exercise-induced increases in DMH CRH (Kawaguchi, Scott et al. 2005). CRH R2, which is highly expressed in the VMN is thought to mediate CRH anorectic effects (Vaughan, Donaldson et al. 1995). CRH R2 expression is reduced in leptin deficient ob/ob mice and in fa/fa Zucker rats that have a dysfunctional leptin receptor (Richard, Rivest et al. 1996; Timofeeva and Richard 1997). Leptin reduces stress-induced activation of the HPA axis as evidenced by reduced cFos activation of PVN CRH neurons and reduced circulating glucocorticoids (Huang, Rivest et al. 1998), but central leptin increases the expression of CRH R2 in the VMN, and it is through this receptor that CRH may have anorexigenic effects (Huang, Timofeeva et al. 2006).

Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors, whose function in the development of LTP (long term potentiation) (Korte, Carroll et al. 1995; Figurov, Pozzo-Miller et al. 1996; Patterson, Abel et al. 1996; Kang, Welcher et al. 1997; Xu, Gottschalk et al. 2000; Zakharenko, Patterson et al. 2003), neuronal survival (Grothe and Unsicker 1987; Hofer and Barde 1988; Kalcheim and Gendreau 1988) and neurogenesis (Alderson, Alterman et al. 1990; Knusel and Hefti 1991) have been well studied. Both BDNF and its receptor tropomyosin-related kinase B (trkB) (Klein, Nanduri et al. 1991) are present in pre-synaptic axon terminals and post-synaptic dendritic compartments, and are capable of bidirectional release and activity (Tyler, Alonso et al. 2002). Central administration of exogenous BDNF

promotes appetite suppression and weight loss (Pelleymounter, Cullen et al. 1995; Wang, Bomberg et al. 2007; Wang, Bomberg et al. 2007; Wang, Godar et al. 2010), and increases locomotor activity (Naert, Ixart et al. 2006) and resting metabolic rate (Wang, Bomberg et al. 2007; Wang, Bomberg et al. 2010) in rodents. In humans, a mutation in the trkB receptor results in severe obesity (Yeo, Connie Hung et al. 2004), suggesting that BDNF effects on energy homeostasis are conserved across species.

In the hypothalamus, exercise and BDNF have anorexigenic effects through seemingly similar mechanisms. RW exercise is a component of environmental enrichment (EE), and it has recently been suggested that EE, including RW, alters food intake and adiposity via a hypothalamic BDNF mediated mechanism (Cao, Choi et al. 2011). Particularly in young animals, EE with RW increases hypothalamic BDNF and is associated with increased sensitivity to leptin and altered synaptic structure of the ARC in favor of increased excitatory input to anorexigenic αMSH neurons and reduced excitatory input to orexigenic AgRP neurons (Mainardi, Scabia et al. 2010). Exercise alone was not sufficient to reduce food intake in the young mice, however RW exercise did increase the anorexigenic effects of leptin (Mainardi, Scabia et al. 2010). Leptin increases BDNF in VMN, DMH and DVC, and in these areas BDNF is associated with reduced feeding (Bariohay, Lebrun et al. 2005; Komori, Morikawa et al. 2006; Wang, Bomberg et al. 2007; Bariohay, Roux et al. 2009; Wang, Bomberg et al. 2010). Yet, the anti-obesity anti-diabetic effects of BDNF on energy metabolism occur even when leptin signaling is impaired, for example in leptin receptor deficient db/db (Tsuchida, Nakagawa et al. 2001; Tsuchida, Nonomura et al. 2001; Tsuchida, Nonomura et al. 2002), and diet induced obese mice (Nakagawa, Ogawa et al. 2003; Tsao, Thomsen et al. 2008). Taken together, BDNF may be an intermediary for leptin-induced reductions in feeding. The PVN contains high levels of BDNF and trkB mRNA (Tapia-Arancibia, Rage et al.

2004). In the PVN, BDNF injections decrease 24-hour food intake and body weight gain and elevate resting metabolic rate and heat production, accompanied by increased uncoupling protein-1 (UCP-1) activity in brown adipose tissue (BAT) (Wang, Bomberg et al. 2007). BDNF increases CRH in the PVN (Jeanneteau, Lambert et al. 2012) (Toriya, Maekawa et al. 2010)), and antagonizing CRHR1 and R2 receptors abolishes BDNF effects on food intake, BW, and body temperature (Toriya, Maekawa et al. 2010). RW exercise also increases PVN CRH expression, and also nearly triples expression of the trkB receptor (Cao, Choi et al. 2011). BDNF is essential for MC4R anorexigenic effects (Xu, Goulding et al. 2003) (Nicholson, Peter et al. 2007) and in the absence of MC4R, exercise attenuates hyperphagia and BW gain (Irani, Xiang et al. 2005; Haskell-Luevano, Schaub et al. 2009). Thus both exercise and BDNF rescue MC4R- knockout-associated hyperphagia and obesity. In addition to affecting feeding behavior, both BDNF (Wang, Bomberg et al. 2007) and exercise (4 weeks) (Cao, Choi et al. 2011) increase UCP-1 and therefore the metabolic activity of BAT, promoting reduced adiposity. Yet after 3 weeks of RW access, there were no changes in BDNF expression in VMN, DMH, PVN, or LH of DIO rats compared with sedentary animals (Patterson, Dunn-Meynell et al. 2008). This does not exclude the possibility that BDNF is involved in hypothalamic plasticity/structural changes at the onset of exercise training. It is also possible that BDNF originally expressed in extra-hypothalamic areas is trafficked to the hypothalamus during wheel running, as BDNF is capable of retrograde and anterograde transport (Nawa, Carnahan et al. 1995; Altar, Cai et al. 1997; Conner, Lauterborn et al. 1997).

Perspectives and Significance

The paradoxical effects of exercise on the central regulation of feeding, where animals either reduce, or fail to increase food intake to compensate for energy used during exercise, are particularly pronounced in DIO, previously DIO and obesity prone animals. A gender dimorphism is also apparent, as females tend to maintain or even increase feeding in response to the metabolic demands of exercise. Future research investigating the gender dimorphism, and possible contributions of sex hormones to exercise effects on appetite are needed. Though the mechanism by which exercise reduces feeding behavior in some rodent models has not been fully elucidated, exercise appears to enhance signaling of leptin, insulin and/or IL-6 receptor, potentially via AMPK. Nutrient sensing may also play a role, as exercise affects substrate utilization and circulating metabolites. One likely contributor to the anorexigenic effects of exercise is signaling of the CRHR2, since exercise anorexigenic effects are abolished with antagonism of this receptor and leptin increases CRHR2 expression in the VMN. BDNF may play a role in mediating exercise anorexigenic effects, as both BDNF and exercise act independently of MC4R signaling to reduce feeding behavior. Further research to elucidate the mechanisms by which exercise affects feeding behavior is relevant to aid in the effective targeting of public health initiatives toward prevention or treatment of obesity.

Chapter 3

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Chapter 3
The lighter side of BDNF

Introduction

Brain Derived Neurotrophic Factor (BDNF) is a member of the neurotrophin family of growth factors (Leibrock, Lottspeich et al. 1989), along with nerve growth factor (Levi-Montalcini 1987), and neurotrophin (NT) 3 (Ernfors, Ibanez et al. 1990; Maisonpierre, Belluscio et al. 1990), NT 4/5 (Berkemeier, Winslow et al. 1991) and NT 6 (Gotz, Koster et al. 1994). Neurotrophins are synthesized as 32-35 kDa pro-isoforms, which are later cleaved to mature forms that dimerize after translation and then act as receptor ligands (Kolbeck, Jungbluth et al. 1994). Whereas the precursor forms of other neurotrophins are constitutively secreted, the 32 kDa pro-BDNF is packaged into vesicles of a regulated pathway and is secreted in an activity dependent manner (Goodman, Valverde et al. 1996). Pro-BDNF may be secreted as is (Chen, Patel et al. 2004), cleaved by the extracellular protease plasmin (Pang, Teng et al. 2004), or interact with the pan-neurotrophin receptor $p75^{NTR}$ and other receptors to have an independent biological effect (Teng, Teng et al. 2005). Alternatively, pro-BDNF is processed to the mature form intracellularly by furin or proconvertases, where it forms C-terminal dimers (Radziejewski, Robinson et al. 1992; Seidah, Benjannet et al. 1996).

Mature BDNF is considered the biologically active form, which has a high affinity for the tropomyosin-related kinase B (trkB) receptor (Klein, Nanduri et al. 1991). Both BDNF and trkB are present in pre-synaptic axon terminals and post-synaptic dendritic compartments of neurons, and are capable of bi-directional release and activity (for review see Tyler 2002) (Tyler, Alonso et al. 2002). Typical of the neurotrophic factors, BDNF stimulates the development and

differentiation of new neurons (Alderson, Alterman et al. 1990; Knusel and Hefti 1991), and promotes long-term potentiation (LTP) (Korte, Carroll et al. 1995; Korte, Staiger et al. 1996; Patterson, Abel et al. 1996), and neuron survival (Grothe and Unsicker 1987; Hofer and Barde 1988; Kalcheim and Gendreau 1988). BDNF is abundantly expressed throughout the developing and mature CNS and in many peripheral tissues such as muscle, liver, and adipose (Lommatzsch, Braun et al. 1999; Cassiman, Denef et al. 2001; Mousavi and Jasmin 2006; Ukropec, Ukropcova et al. 2008). Regional differences between BDNF mRNA levels and protein concentrations in the CNS are often reported (Nawa, Carnahan et al. 1995; Narisawa-Saito, Wakabayashi et al. 1996; Altar, Cai et al. 1997; Conner, Lauterborn et al. 1997), which may be related to regulatory mechanisms, mRNA decay (Malter 2001), or BDNF anterograde transport (Altar, Cai et al. 1997).

BDNF is synthesized in several areas of the hypothalamus, including the paraventricular nucleus (PVN), the ventromedial hypothalamic nucleus (VMN), the dorsomedial hypothalamic nucleus (DMN), and the lateral hypothalamic area (LH) (Conner, Lauterborn et al. 1997). Additionally BDNF immunoreactive fibers have been identified in the arcuate nucleus (Arc), however the Arc does not appear to be a site of BDNF synthesis (Conner, Lauterborn et al. 1997). *BDNF* is also widely expressed throughout the hippocampus, amygdala, select areas of the thalamus including the ventral tegmental area (VTA), and in areas of the hindbrain including the dorsal vagal complex (DVC) (Conner, Lauterborn et al. 1997; Bariohay, Lebrun et al. 2005). In recent years much attention has been given to BDNF for its role in energy homeostasis. While examining BDNF and neuronal plasticity *in vivo*, Lapchak *et al*. noted that chronic intraventricular (icv) administration of BDNF prevented weight gain (Lapchak and Hefti 1992). It has since been observed in many studies that central administration of BDNF induces appetite suppression and weight loss (Pelleymounter, Cullen et al. 1995; Wang,

Bomberg et al. 2007; Wang, Bomberg et al. 2007; Wang, Godar et al. 2010), increases locomotor activity (Naert, Ixart et al. 2006), and resting metabolic rate (Wang, Bomberg et al. 2007; Wang, Bomberg et al. 2010). An obese phenotype is also observed in BDNF conditional knockout mice, where BDNF is deleted after birth and the knockout is restricted to the brain (Rios, Fan et al. 2001). In addition to central effects, BDNF exerts peripheral actions that affect glucose metabolism (Yamanaka, Tsuchida et al. 2007; Yamanaka, Itakura et al. 2008; Yamanaka, Itakura et al. 2008), energy expenditure (Yamanaka, Itakura et al. 2007; Yamanaka, Tsuchida et al. 2007), and food intake (Yamanaka, Itakura et al. 2008). Both central and peripherally administered BDNF lowers blood glucose and increases energy expenditure in animal models of type-2 diabetes (Nakagawa, Tsuchida et al. 2000). The combined effects of central and peripheral BDNF are apparent from instances where BDNF is globally reduced, as is the case in rodents and human subjects with haploinsufficiency for the gene, which results in obesity and hyperphagia (Kernie, Liebl et al. 2000; Gray, Yeo et al. 2006; Han, Liu et al. 2008). The neurotrophin receptor trkB is a receptor tyrosine kinase, which upon activation results in receptor dimerization, followed by receptor trans-phosphorylation and the initiation of intracellular signaling cascades. A human mutation affecting the ability of the trkB receptor to autophosphorylate is associated with obesity and hyperphagia (Yeo, Connie Hung et al. 2004). The trkB receptor exists in full length and two truncated forms, but only the full-length receptor contains intracellular tyrosine kinase activity (Allendoerfer, Cabelli et al. 1994). The two truncated forms are generated by alternative splicing of the full-length receptor, and are capable of inhibiting activity of BDNF by forming heterodimers with full length trkB (Eide, Vining et al. 1996), or by binding and internalizing BDNF (Haapasalo, Sipola et al. 2002). The role of BDNF in obesity appears to, at least in part, involve signaling through the full-length trkB receptor, as trkB hypomorphs, expressing ¼ of the full-length trkB

receptor of wild type mice, are obese (Xu, Gottschalk et al. 2000; Xu, Goulding et al. 2003). In the absence of BDNF the anti-obesity effect of trkB is still possible. This is evidenced by the use of two different BDNF agonists, the trkB ligand NT4 and a trkB specific antibody that acts as a receptor agonist, which when administered to the hypothalamus of mice caused reductions in food intake and resistance to diet induced (DRO), polygenic, and leptin receptor deficiency associated obesity (Tsao, Thomsen et al. 2008). It is important to note that BDNF appears to be the main natural ligand for trkB, as rodents heterozygous for BDNF, but not NT4, display the obese phenotype (Kernie, Liebl et al. 2000). Thus both BDNF and trkB are necessary for BDNF-mediated effects on energy balance (Tsao, Thomsen et al. 2008).

The human *BDNF* gene contains a total of 10 exons coding for the 5' untranslated region, and are alternatively spliced to a common 3' coding exon, resulting in 34 possible mRNA transcripts (Pruunsild, Kazantseva et al. 2007). In rodents *BDNF* contains 9 exons, encoding for 24 different mRNA transcripts, each of them ultimately translating into an identical mature BDNF (reviewed by Cunha 2010) (Cunha, Brambilla et al. 2010). The expression of *BDNF* transcripts is tissue specific, differentially expressed throughout different brain sites and peripheral tissues (Timmusk, Palm et al. 1993; Bishop, Mueller et al. 1994). Moreover, environmental cues, such as stress, can alter transcript expression (Marmigere, Givalois et al. 2003; Fuchikami, Morinobu et al. 2009; Tognoli, Rossi et al. 2010), which is associated with alterations in pro-BDNF/total BDNF ratio (Tognoli, Rossi et al. 2010). The exact role of each of these individual transcripts is still largely unknown. In the VMN of rodents, transcripts encoding for *BDNF* exons I, II and IV are expressed, whereas exon III is not (Tran, Akana et al. 2006). Transcription of exons I and IV in this region are regulated by steroidogenic factor 1 (SF-1), which, when reduced (as in the case of SF-1 heterozygotes), impairs hypothalamic function and results in hyperphagia and

weight gain (Tran, Akana et al. 2006). Different genetic variants impact the activity of BDNF by affecting biosynthesis and/or post-translational processing of the pro-BDNF precursor (Mowla, Farhadi et al. 2001). One variant in particular, the SNP that results in a substitution of a valine for methionine residue at position 66 (Val66Met), has been identified as having a strong correlation with eating disorders (ED), specifically anorexia nervosa (AN) of the restricting type, and is associated with low BMI (Ribases, Gratacos et al. 2003). This polymorphism alters the intracellular packaging of pro-BDNF and may affect activity-dependent secretion of the mature BDNF peptide (Egan, Kojima et al. 2003; Chen, Patel et al. 2004). According to a recent meta-analysis, the Val66Met polymorphism is associated with a 33% increased risk for ED (Gratacos, Gonzalez et al. 2007), however this analysis did not subdivide ED by category. Recent evidence is conflicted regarding this gene variant, as some have found no association between ED and Val66Met (Arija, Ferrer-Barcala et al. 2010), or AN (Dardennes, Zizzari et al. 2007), and one study reported a link between Val66Met and obesity in females (Beckers, Peeters et al. 2008). The conflicting evidence regarding ED and the *BDNF* SNP Val66Met reflects both the complexity of eating disorders and the range of factors effecting feeding behavior.

In a recent genome-wide association study, *BDNF* was one of 18 gene loci where having a certain SNP variant was associated with higher BMI (Speliotes, Willer et al. 2010). Another study examined 41 different SNPs near the *BDNF* locus in 87 adults with sudden death, and made comparisons between BMI and BDNF mRNA in the VMN of the cadavers. In the subjects with extreme obesity (BMI ≥40kg/m^2), *BDNF* expression was reduced 2 fold compared with overweight and obese individuals. There was an association between homozygosity for the minor C allele at rs12291063, reduced VMN *BDNF* expression, and high BMI, which suggests that having the SNP at rs12291063 may be a risk factor for obesity (Ong, Han et al. 2010). In mice, heterozygosity for *Bdnf* decreases

hypothalamic expression and results in hyperphagia and obesity (Kernie, Liebl et al. 2000). WAGR (Wilms' tumor, aniridia, genitourinary anomalies and mental retardation) syndrome is a rare disorder characterized by heterozygous gene deletions in at least two genes located near *BDNF* in the 11p13 region, and sometimes accompanies the heterozygous deletion of *BDNF.* In a study of individuals with WAGR syndrome, 100% of those heterozygous for *BDNF* deletion were obese by the age of 10, in contrast with 20% of those without *BDNF* deletion (Han, Liu et al. 2008).

BDNF and the central regulation of energy metabolism

BDNF was first observed to affect energy metabolism with icv administration (Pelleymounter, Cullen et al. 1995). In recent years, studies have identified additional sites of BDNF action regulating energy balance, including the DVC, hypothalamic PVN and VMN, the VTA, amygdala, and possibly the hippocampus (Bariohay, Lebrun et al. 2005; Davidson, Kanoski et al. 2005; Wang, Bomberg et al. 2007; Wang, Bomberg et al. 2007; Wang, Bomberg et al. 2007; Bariohay, Roux et al. 2009; Davidson, Chan et al. 2009; Boghossian, Park et al. 2010; Cordeira, Frank et al. 2010; Wang, Bomberg et al. 2010; Wang, Godar et al. 2010; Wang 2010). In regulating energy balance BDNF interacts with several other neuropeptides, including melanocortin (Xu, Goulding et al. 2003; Nicholson, Peter et al. 2007; Tsao, Thomsen et al. 2008; Bariohay, Roux et al. 2009; Cao, Lin et al. 2009), leptin (Bariohay, Lebrun et al. 2005; Komori, Morikawa et al. 2006; Wang, Bomberg et al. 2007; Bariohay, Roux et al. 2009; Cao, Liu et al. 2010; Wang, Bomberg et al. 2010; Wang 2010), corticotrophin-releasing hormone (CRH) (Wang 2008; Byerly, Simon et al. 2009; Cao, Lin et al. 2009; Cao, Liu et al. 2010; Toriya, Maekawa et al. 2010) and thyrotropin releasing hormone (TRH) (Smith, Makino et al. 1995; Ubieta, Uribe et al. 2007; Byerly, Simon et al. 2009).

In 1995, Pelleymounter *et al.* discovered that icv administration of BDNF decreased energy intake and body weight of rats, which was associated with a dose dependent increase in serotonin turnover (Pelleymounter, Cullen et al. 1995). A pair-fed group had comparable weight loss, but the recovery weight gain in these rats was much faster than the BDNF infused group (Pelleymounter, Cullen et al. 1995), suggesting BDNF promotes lasting metabolic changes. Additional evidence for a central role of BDNF in the regulation of energy balance is apparent, as animals with reduced *Bdnf* expression, either due to a conditional homozygous knockout in the brain or due to heterozygous gene expression (*Bdnf* $^{+/}$), develop hyperphagia, obesity, and resistance to insulin and leptin (Kernie, Liebl et al. 2000; Rios, Fan et al. 2001). Administration of BDNF icv reverses the hyperphagic and obese phenotype of *Bdnf* $^{+/}$mutant mice (Kernie, Liebl et al. 2000). A single icv injection of BDNF is sufficient to improve insulin receptor signaling in the liver of STZ-induced diabetic mice, whereas no direct effect of BDNF on cultured hepatocytes has been observed (Tsuchida, Nakagawa et al. 2001). This indicates that independently of anorectic effects, centrally administered BDNF may affect glucose metabolism. Nonomura *et al.* observed that icv BDNF dose-dependently lowers blood glucose and increases pancreatic insulin content in leptin receptor deficient db/db mice, and does so independently of food intake. These improvements were also associated with increased norepinephrine turnover and uncoupling protein-1 (UCP-1) expression in brown adipose tissue (BAT) (Nonomura, Tsuchida et al. 2001). Taken together it appears the central BDNF enhances energy expenditure via activation of the sympathetic nervous system and improves blood glucose in obese, diabetic rodents. It should be noted that BDNF does not affect blood glucose in normoglycemic rats (Pelleymounter, Cullen et al. 1995).

The adult rat hypothalamus contains high levels of BDNF (Nawa, Carnahan et al. 1995; Katoh-Semba, Takeuchi et al. 1997; Tapia-Arancibia,

Rage et al. 2004), and most hypothalamic neurons express the trkB receptor (Merlio, Ernfors et al. 1992; Castren, Thoenen et al. 1995; Marmigere, Rage et al. 1998). Overexpression of the *Bdnf* gene in the hypothalamus is associated with increased heat production, respiratory exchange ratio and resting metabolism, and increased hypothalamic expression of trkB, insulin receptor, CRH and TRH (Cao, Lin et al. 2009; Cao, Liu et al. 2010). Increased *Bdnf* expression is also associated with sharp decreases in leptin and insulin concentrations, and increases in the adipose tissue-secreted hormone adiponectin (Cao, Lin et al. 2009), which is associated with increased fatty acid oxidation, enhanced glucose metabolism and weight loss (Fruebis, Tsao et al. 2001). We have found that BDNF reduces food intake and influences energy expenditure when injected into certain specific hypothalamic sites, but not others. For example, although the LH expresses BDNF and its receptor, specific site injection of BDNF did not significantly reduce feeding and body weight (Wang, Bomberg et al. 2007). The hypothalamic PVN is responsive to physiological stimuli, is involved in stress responses (Bartanusz, Jezova et al. 1993; Givalois, Arancibia et al. 2000), and contains high levels of BDNF and trkB mRNA (Tapia-Arancibia, Rage et al. 2004). We found that injections of BDNF in the PVN increases energy expenditure, mainly by increasing resting metabolic rate (RMR), and increasing thermogenic capacity as indicated by elevation of UCP-1 expression in BAT (Wang, Bomberg et al. 2007). We have also found that a single injection of BDNF reduces food intake and body weight (Wang, Bomberg et al. 2007) for up to 48 hours after injection, suggesting a prolonged and potent effect. Animals who were made obese with a high fat (HFD) and subsequently given PVN injections of BDNF on alternate days over an extended period, had significant reductions in energy intake, body weight, and body fat (including visceral fat) compared with aCSF-injected controls. BDNF also normalized HFD-induced hyperglycemia, hyperlipidemia, hyperinsulinemia and hyperleptinemia, suggesting chronic BDNF

in the PVN improves metabolic syndrome and associated resistance to insulin and leptin (Wang, Godar et al. 2010). Furthermore, the animals more susceptible to DIO were also more sensitive to PVN injections of BDNF (Wang, Godar et al. 2010).

Stress paradigms increase expression of BDNF mRNA in the PVN (Rage, Givalois et al. 2002), which is associated with decreased inhibitory synaptic input leading to activation of PVN neurons (Verkuyl, Hemby et al. 2004; Verkuyl, Karst et al. 2005). The mechanism for BDNF-associated removal of inhibition was elucidated by Hewitt and Bains, who observed that through trkB receptor activation on postsynaptic neurons, BDNF reduces the surface expression of inhibitory $GABA_A$ receptor clusters in the PVN (Hewitt and Bains 2006). Thus, it is likely that BDNF increases the firing rate of PVN neurons involved in the stress response. CRH neurons in the PVN regulate locomotor activity and body temperature (Rowsey and Kluger 1994; Linthorst, Flachskamm et al. 1997), and thus the removal of inhibitory $GABA_A$ receptors on these neurons would likely account for the physiological and behavioral effects observed when BDNF is injected directly in this area. Naert *et al.* observed that BDNF infused continuously into the lateral ventricle of rats causes a significant increase in paraventricular CRH and AVP mRNA, which is associated with increased locomotor activity, body temperature, and reduced body weights (Naert, Ixart et al. 2006). BDNF over-expression in the hypothalamus also resulted in increased CRH expression (Cao, Lin et al. 2009), and hypothalamic increases in BDNF and CRH were associated with β-adrenergic receptor activation (Cao, Liu et al. 2010). Again, these data support the notion that the sympathetic nervous system is important in BDNF-associated metabolic changes. Toriya *et al.* report that PVN-injected BDNF-induced reduction in feeding and body weight is mediated via CRH-R2, and accordingly the effects of BDNF were blocked using a CRH antagonist (Toriya, Maekawa et al. 2010). Similarly, we have observed that the

effect of BDNF on feeding and body weight gain due to BDNF injections in the VMN or PVN was attenuated by pre-treatment with a CRH antagonist (Wang 2008). In the PVN, mRNA for BDNF and CRH are co-localized (Naert, Ixart et al. 2006), and there is also co-localization for trkB receptor and CRH (Toriya, Maekawa et al. 2010), suggesting potential signaling between the two neuropeptides in the regulation of energy metabolism. (Figure 1)

The PVN is a site for TRH synthesis and secretion, and TRH plays an important role in the control of energy homeostasis (Lechan and Fekete 2006) through the TRH-TSH-TH cascade. Triiodothyronine (T_3) activates orexigenic neurons of the VMN, and stimulates food intake independently of energy expenditure (Kong, Martin et al. 2004). TRH neurons express BDNF in response to immobilization stress (Smith, Makino et al. 1995). Interestingly, BDNF and T_3 have opposing effects on the expression of several obesity-related genes in the hypothalamus (Byerly, Simon et al. 2009). BDNF increases, while T_3 decreases expression of BDNF, leptin receptor, pro-opiomelanocortin (POMC), TRH, and agouti-related protein (AgRP) (Byerly, Simon et al. 2009). Through trkB receptor signaling, BDNF increases the expression of the TRH precursor pre-pro-TRH mRNA in PVN neurons (Ubieta, Uribe et al. 2007). Thus in the PVN, BDNF may, in part, contribute to negative energy balance via increasing TRH, or, conversely, TRH may affect energy balance by increasing BDNF.

POMC and AgRP (NPY/AgRP) neurons are two distinct populations of neurons that project from the hypothalamic Arc to the PVN and release alpha-melanocyte stimulating hormone (α-MSH) and AgRP respectively (Fekete, Mihaly et al. 2000; Fekete, Marks et al. 2004), which interact with the melanocortin receptor (MC3/4R) in the PVN to elicit opposing actions on food intake. The MC4R is a membrane-bound α-MSH receptor (O'Rahilly, Yeo et al. 2004), highly expressed in both the hypothalamus and brainstem (Wu, Gao et al. 2004), and is important for maintaining a lean phenotype. Animals heterozygous for, or lacking

the MC4R gene become obese, (Huszar, Lynch et al. 1997), and MC4R agonist infusion reduces food intake and lowers body weight in HFD-fed rats (Seeley, Burklow et al. 2005). Delivery of *BDNF* gene into the hypothalamus increases the expression of MC4R in animals fed with regular chow or a HFD (Cao, Lin et al. 2009), suggesting BDNF affects melanocortin signaling (Cao, Lin et al. 2009). Notably, activation of MC4R leads to acute elevations of hypothalamic BDNF, which is critical for melanocortinergic effects on appetite and body temperature (Nicholson, Peter et al. 2007). BDNF is highly expressed in the VMN (Unger, Calderon et al. 2007), which is an important area for regulating energy metabolism (Sakaguchi, Arase et al. 1988). MC4R controls BDNF expression in the VMN, and infusion of BDNF in the brain of MC4R deficient mice attenuates hyperphagia and excessive weight gain induced by a moderate fat diet (25.1% calories from fat)(Xu, Goulding et al. 2003). In addition, the phenotype of the trkB homomorph, a mutant with reduced BDNF/trkB signaling, is similar to the MC4R-null mutant mouse (Xu, Goulding et al. 2003). The effects of BDNF in MC4R signaling are dependent on trkB activation, as trkB ligands reduce food intake and body weight downstream of MC4R in the hypothalamus of mice (Tsao, Thomsen et al. 2008). Together these data suggest that BDNF is a downstream effector of MC4R activation, and that BDNF-trkB signaling is an essential part of the mechanism for the anorectic and obesity-resistant effects of MC4R agonists in the VMN **(Figure 3.1)**.

The VMN is involved in the control of autonomic responses that contribute to the prevention of obesity (reviewed by King (King 2006)). Neurons of the VMN project to many areas associated with feeding behavior, including the amygdala (Saper, Swanson et al. 1976; Canteras, Simerly et al. 1994), Arc (Sternson, Shepherd et al. 2005), LH (Sclafani, Berner et al. 1975), PVN (Lin and York 2004), the DMN (Luiten and Room 1980; Ter Horst and Luiten 1987), as well as areas relating to rewarding aspects of feeding behavior including the VTA (Saper,

Swanson et al. 1976), the NA, and the nucleus of the solitary tract (Canteras, Simerly et al. 1994). The VMN receives inputs from the Arc (Bagnol, Lu et al. 1999; Haskell-Luevano, Chen et al. 1999), the LH (Saper, Swanson et al. 1976; Ter Horst and Luiten 1987; Fahrbach, Morrell et al. 1989), and the amygdala (Luiten, Ono et al. 1983; Martinez-Marcos, Lanuza et al. 1999). The VMN is involved in promoting satiety, as lesions in this area are associated with hyperphagic behavior (King, Phelps et al. 1980). Steroidogenic factor 1 (SF-1) is a nuclear hormone receptor important to the developmental structure of the VMN (Ikeda, Luo et al. 1995; Shinoda, Lei et al. 1995). SF-1 is co-expressed with BDNF in the VMN and is involved in BDNF synthesis. Reduced levels of SF-1 as seen in heterozygous animals are associated with reduced BDNF, increased weight gain, hyperphagia, and lower daytime metabolic rate (Tran, Akana et al. 2006).

We found that a single injection of BDNF directly into the VMN, at doses not causing taste aversion, significantly decreases normal feeding and deprivation-and NPY-induced feeding for up to 48 hours (Wang, Bomberg et al. 2007). No effects on feeding behavior were observed during the initial 4 hours post injection, indicating that the feeding effects of injected BDNF might be indirect, or might take place as a result of retrograde (Mufson, Kroin et al. 1994; Mufson, Kroin et al. 1996) or anterograde (Sobreviela, Pagcatipunan et al. 1996; Altar, Cai et al. 1997; Conner, Lauterborn et al. 1997) transfer to a different brain location (Wang, Bomberg et al. 2007). The possibility that BDNF may act locally to reduce food intake by altering synaptic strength or receptor expression in the VMN has not been adequately investigated, but is worth considering. In contrast to the delayed anorectic action, BDNF in the VMN immediately increased energy expenditure by elevating resting metabolic rate and physical activity (Wang, Bomberg et al. 2010). Chronic BDNF in the VMN also reduces HFD-induced obesity by reducing energy intake and/or increasing energy expenditure based

on the phenotype of the animals on a HFD (Wang 2010). Unlike in the PVN, BDNF in the VMN significantly increases physical activity in animals on regular chow (Wang, Bomberg et al. 2010) or HFD, suggesting that elevated physical activity-induced energy expenditure contributes to increased thermogenesis induced by BDNF in the VMN. BDNF in the VMN also decreases respiratory exchange ratio in animals on regular chow (Wang, Bomberg et al. 2010) and a HFD (Wang 2010), indicating that BDNF stimulates fat metabolism, which partially explains the preferential loss of fat (vs. lean) mass after BDNF treatment. Deletion of BDNF in the VMN causes hyperphagia and obesity in mice (Unger, Calderon et al. 2007), further confirming the importance of BDNF as a contributor to the maintenance of a lean phenotype. BDNF expression is responsive to dietary cues, as transcriptional levels of BDNF in the VMN are reduced by fasting (Xu, Goulding et al. 2003) and elevated by glucose (Unger, Calderon et al. 2007). It is possible that HFD induced obesity results from lack of responsiveness to these dietary cues. In support of this, Yu *et al.* observed decreased BDNF mRNA in the VMN of diet induced obese mice, compared with mice resistant to HFD-induced obesity (Yu, Wang et al. 2009).

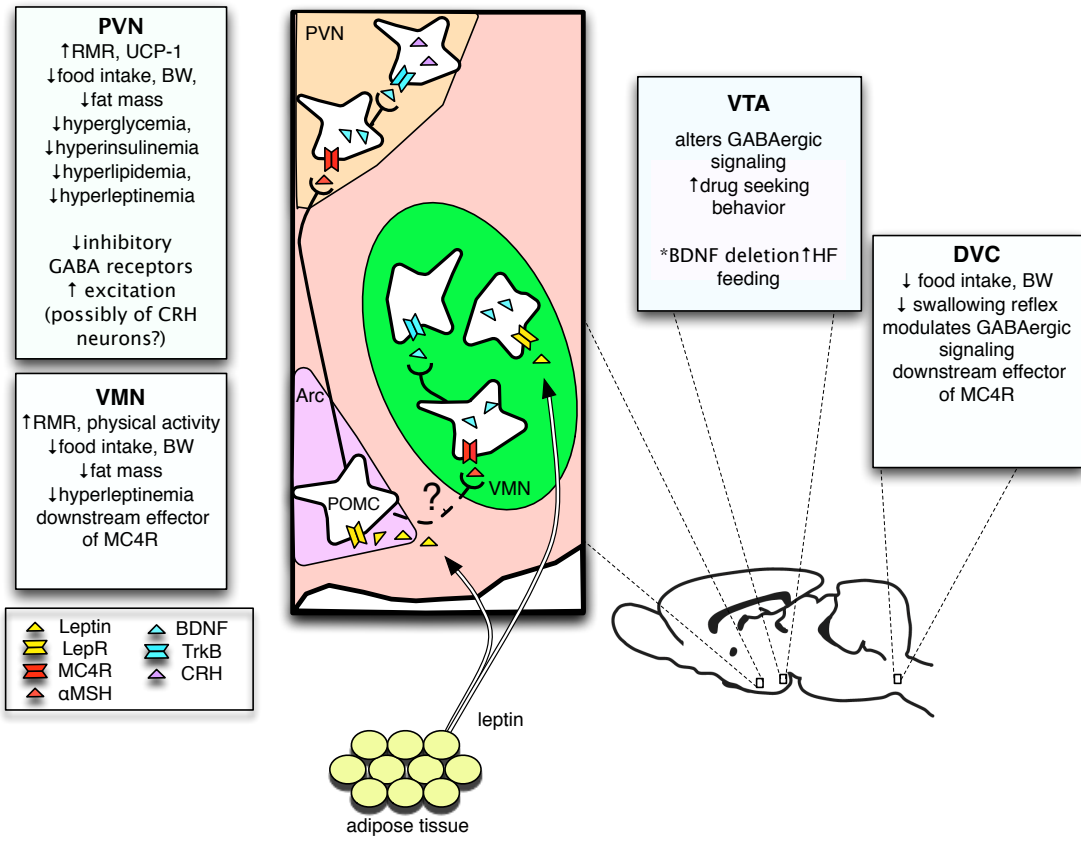

Figure 3.1 BDNF and the central regulation of energy balance

Leptin is secreted from adipose tissue and activates receptors of the anorexigenic pro-opiomelanocortin (POMC) neurons of the arcuate nucleus (Arc) and neurons of the ventromedial nucleus of the hypothalamus (VMN). POMC neurons project to the hypothalamic paraventricular nucleus (PVN) and VMN where they secrete alpha-menalocyte stimulating hormone (α-MSH), which binds to the melanocortin receptor (MC4R). MC4R activation in the VMN controls BDNF expression, however arcuate POMC neurons project sparsely to the VMN, and it is currently unknown whether α-MSH coming from these projections affects BDNF expression (Xu, Goulding et al. 2003). Both leptin and α-MSH induce the expression of brain derived neurotrophic factor (BDNF) in the VMN. BDNF activates the tropomyosin-related kinase B (TrkB) receptor in neurons of the VMN and PVN with consequences for energy metabolism

(boxes on the left). In PVN neurons, BDNF removes inhibitory $GABA_A$ receptor clusters, allowing for greater neuronal excitability. Neurons of the PVN express corticotropin-releasing hormone (CRH) and in the PVN effects of BDNF are attenuated when the CRH receptor is blocked using a receptor antagonist, indicating that BDNF activates the CRH-urocortin-CRH-R2 pathway. BDNF is expressed in the dorsal vagal complex (DVC) (box on the right), where it regulates energy metabolism as a downstream effector of the MC4R. BDNF is also expressed in the ventral tegmental area (VTA) where it is possibly involved in hedonic aspects of feeding (center box). Inset figure adapted from Paxinos and Watson (Paxinos and Watson 2007).

The adipokine leptin is produced and released from adipose tissue. Leptin signaling in the CNS inhibits food intake and increases energy expenditure, and in so doing, counters the accumulation of adiposity. Of note, leptin increases BDNF in certain brain areas, including the DVC (Bariohay, Lebrun et al. 2005), VMN and DMN (Komori, Morikawa et al. 2006), with reductions in food intake (Bariohay, Lebrun et al. 2005; Komori, Morikawa et al. 2006; Wang, Bomberg et al. 2007; Bariohay, Roux et al. 2009; Wang, Bomberg et al. 2010) (figure1). However, the anti-obesity anti-diabetic effects of BDNF on energy metabolism occur downstream of leptin signaling, as the effects are observed in leptin receptor deficient db/db (Tsuchida, Nakagawa et al. 2001; Tsuchida, Nonomura et al. 2001; Tsuchida, Nonomura et al. 2002), Kkay (an animal model of metabolic syndrome) (Nakagawa, Ogawa et al. 2003), and diet-induced obese

(DIO) mice (Nakagawa, Ogawa et al. 2003; Tsao, Thomsen et al. 2008). Since leptin receptor signaling is important for leptin-dependent BDNF up-regulation (Komori, Morikawa et al. 2006), the obese phenotype might partially be related to the inability of leptin to increase hypothalamic BDNF expression. While leptin increases hypothalamic BDNF, BDNF decreases leptin production in adipocytes, an effect which involves sympathoneural beta-adrenergic signaling and the hypothalamic-pituitary-adrenal axis (HPA) (Cao, Liu et al. 2010). HFD induces leptin resistance, characterized as a reduced anorectic response to leptin, as well as hyperleptinemia. We found that VMN BDNF significantly attenuated hyperleptinemia compared to that prior to BDNF intervention, or to vehicle-treated control animals (Wang 2010) on a HFD. Additional studies are needed to explore whether the observed attenuated hyperleptinemia after BDNF is associated with or is an indication of improved leptin sensitivity.

Both BDNF and the trkB receptor are highly expressed in the DVC of the hindbrain (Conner, Lauterborn et al. 1997). The DVC is located in the caudal brainstem, an autonomic integrator of food intake control (Bariohay, Lebrun et al. 2005), and is involved in integrating satiety signals emanating from peripheral fat stores (Lebrun, Bariohay et al. 2006). BDNF acts as an anorexigenic factor in the DVC; Bariohay *et al*. reported that BDNF infusion in the DVC induced anorexia and weight loss (Bariohay, Lebrun et al. 2005). Noteworthy, the efficacy of BDNF as an anorectic agent in the DVC decreased over a 14-day infusion period, indicating some compensation or desensitization occurs. The protein content of BDNF in the DVC decreases after 48 hours of food deprivation and increases upon refeeding. Furthermore, the anorexigenic hormones leptin and cholecystokinin (CCK) injected peripherally increase BDNF content in the DVC (Bariohay, Lebrun et al. 2005). The DVC contains the neural network responsible for the central pattern generator of swallowing (Jean 2001) and BDNF-trkB signaling inhibits the swallowing reflex via modulation of GABAergic signaling

(Bariohay, Tardivel et al. 2008). Similarly, increased stimulation of the superior laryngeal nerve decreases BDNF in the DVC, indicating positive feedback allowing the swallowing reflex to continue with the presence of a food stimulus (Lebrun, Bariohay et al. 2006; Bariohay, Tardivel et al. 2008). BDNF is a downstream effector of the MC4R signaling pathway in the DVC, and is necessary for the anorexigenic effect of MC4R activation (Bariohay, Roux et al. 2009). The orexigenic effect of an MC4R antagonist is abolished with co-administration of BDNF, and pharmacological blockade of the trkB receptor attenuates the anorexigenic effect of an MC4R agonist (Bariohay, Roux et al. 2009). Taken together, BDNF in the DVC appears to be responsive to hormonal satiety signals as well as physical signals of the presence of a food stimulus, and coordinates swallowing (**Fig. 3.1**).

MC4R is expressed in the amygdala (Mountjoy, Mortrud et al. 1994) an area involved in regulating macronutrient selection (King, Rossiter et al. 1998) and some reward aspects of feeding behavior (Kelley 2004; Figlewicz, MacDonald Naleid et al. 2007). In the amygdala, injection of MC4R agonist causes a dose dependent reduction in food intake, which is greater in animals fed a HFD (Boghossian, Park et al. 2010). Surprisingly, while injections of the orexigenic hormone AgRP in the amygdala increases food intake, it is also associated with elevated amygdala BDNF mRNA (Boghossian, Park et al. 2010). This effect is unexpected and warrants further investigation, as compelling evidence suggests that BDNF is a downstream effector of anorexigenic melanocortinergic signaling (Boghossian, Park et al. 2010).

BDNF and trkB are expressed in the mesolimbic dopamine system, which is associated with hedonic reward (Seroogy, Lundgren et al. 1994; Numan and Seroogy 1999). The consumption of palatable high fat foods alters the expression of BDNF and trkB receptor in the VTA, but not the nucleus accumbens (NAc) of wild type mice (Cordeira, Frank et al. 2010). BDNF is not highly expressed in the

NAc, so most of the BDNF found there is produced in the VTA and anterogradely transported from neurons that originate there (Conner, Lauterborn et al. 1997; Numan and Seroogy 1999). The neurons of the NAc release dopamine in response to palatable foods (Bassareo and Di Chiara 1997). Site-specific viral depletion of BDNF in the VTA causes excessive intake of a palatable HFD, but not standard chow, whereas reduced BDNF in the VMN results in indiscriminate hyperphagia of either HFD or chow (Unger, Calderon et al. 2007; Cordeira, Frank et al. 2010). Peripheral administration of a D_1 receptor agonist normalizes the caloric intake of palatable HFD in BDNF mutant mice (Cordeira, Frank et al. 2010), indicating that BDNF synthesis in the VTA is possibly involved in dopamine secretion from neurons of the NAc and thus BDNF may play a role in hedonic reward (**Fig. 3.1**)

The hippocampus, which has long been associated with learning and memory (Squire 1992; Jarrard 1995), has been implicated in having a potential involvement in energy balance (Davidson, Kanoski et al. 2005). Part of what makes this an attractive hypothesis is that areas of the hippocampus, in particular, field CA1 neurons, project to the LH, Arc, PVN, DMN and VMN, all important hypothalamic areas involved in feeding behavior (Cenquizca and Swanson 2006). Several multisynaptic pathways have been identified that connect brainstem feeding control areas to the hippocampus (Moser and Moser 1998; Grill and Kaplan 2002). Amnesic patients with hippocampus damage showed reduced sensitivity to interoceptive signals of hunger and satiety (Hebben, Corkin et al. 1985; Rozin, Dow et al. 1998). Compared to intact controls, rodents with selective lesions of the hippocampus exhibit increased appetitive responding for food (Davidson, McKernan et al. 1993; Schmelzeis and Mittleman 1996; Clifton, Vickers et al. 1998). In a recent study by Davidson *et al.*, lesioning of the complete hippocampus resulted in increased food intake, body weight gain, appetitive behavior and metabolic activity (Davidson, Chan et al.

2009). When lesioning was restricted to the ventral pole, which projects to the lateral hypothalamus (Cenquizca and Swanson 2006), animals had increased food intake and body weight (Davidson, Chan et al. 2009). Functional magnetic resonance imagery (fMRI) imaging identified the hippocampus and prefrontal cortex as the sites of greatest activation in obese people (Wang, Yang et al. 2006). DelParigi *et al*. also noticed a decreased hippocampal blood flow in obese and formerly obese people after they consumed a liquid meal to satiation (DelParigi, Chen et al. 2004). These findings suggest that the hippocampus plays an important role in the regulation of energy metabolism. BDNF and trkB are highly expressed in the hippocampus. Many studies have reported that exercise increases hippocampal BDNF expression (Chen and Russo-Neustadt 2009; Griffin, Bechara et al. 2009; Seifert, Brassard et al. 2010), and that these increases are associated with enhanced cognition (Nichol, Deeny et al. 2009; Berchtold, Castello et al. 2010; Lafenetre, Leske et al. 2010). Exactly what role, if any, hippocampal BDNF plays in energy metabolism is still unclear. Some evidence suggests that hippocampal BDNF might be related to factors affecting the memory of food, and therefore motivation to eat (Gelegen, van den Heuvel et al. 2008). A/J mice, who behaviorally model activity-induced anorexia and exhibit reduced food anticipatory activity, have significantly lower BDNF expression in the hippocampus during feeding times compared with mice that have normal food anticipatory activity (Gelegen, van den Heuvel et al. 2008). Dietary restriction has no effect on hippocampal BDNF in A/J mice, whereas in mice without activity-induced anorexia, dietary restriction increases hippocampal BDNF (Lee, Seroogy et al. 2002; Duan, Guo et al. 2003; Gelegen, van den Heuvel et al. 2008), as well as increasing the full-length trkB receptor (Lee, Seroogy et al. 2002). This is likely not directly due to altered glucose levels, as no changes in BDNF expression were observed in the hippocampus with icv glucose administration (Unger, Calderon et al. 2007). A HFD decreases hippocampal BDNF (Wu, Molteni et al.

2003; Molteni, Wu et al. 2004; Wu, Ying et al. 2004; Wu, Hu et al. 2006; Park, Park et al. 2010), as does a HF-high sugar diet (Molteni, Barnard et al. 2002; Kanoski, Meisel et al. 2007; Stranahan, Norman et al. 2008), however the type of sugar matters as the combination of a HFD and dextrose decreased BDNF whereas HFD and sucrose did not. In this study, rats gained similar amounts of weight, indicating the differences in BDNF expression were not directly related to body weight (Kanoski, Meisel et al. 2007). Furthermore, a HFD does not always decrease hippocampal BDNF. In an animal model of early life trauma, where rats were separated from dams for about 2 weeks after birth, a HFD was associated with increased hippocampal BDNF (Maniam and Morris 2010). Yu *et al.* observed that in animals prone to DIO, hippocampal BDNF was reduced in response to a HFD, whereas in obesity resistant animals (DRO), or in pair fed DIO animals it was not. The authors speculate that the decreases in hippocampal BDNF signaling may correspond to weakened inhibitory control of HF food intake and promote obesity (Yu, Wang et al. 2009). Davidson *et al*. proposed a "vicious circle" model: an unhealthy diet (such as HFD) reduces hippocampal BDNF, which causes hippocampal dysfunction (such as hypermnesia), which may result in impaired feeding behavior and overeating in an "obesigenic" environment, which further reduces hippocampal BDNF and damages hippocampal function, and continues the circle (Davidson, Kanoski et al. 2005).

Peripheral actions of BDNF

In addition to the brain, BDNF is expressed in many tissues important to the regulation of energy homeostasis, namely adipose tissue, skeletal and smooth muscle, and liver (Lommatzsch, Braun et al. 1999; Cassiman, Denef et al. 2001; Mousavi and Jasmin 2006; Ukropec, Ukropcova et al. 2008). It is therefore important to consider that the effect of BDNF in these peripheral tissues might also contribute to the overall maintenance of energy balance.

BDNF mRNA and protein expression is increased in exercising skeletal muscle, an effect associated with the phosphorylation of AMP-activated protein kinase (AMPK) and acetyl-coA carboxylase β (ACCβ), as well as increases in fatty acid oxidation (Matthews, Astrom et al. 2009). AMPK "senses" a high-energy state in muscle, and when activated it phosphorylates the mitochondrial ACCβ, thereby inhibiting increases in malonyl-CoA levels (an action that ultimately leads to increased mitochondrial fatty acid transport and oxidation) (McGarry, Takabayashi et al. 1978; Carling, Zammit et al. 1987; McGarry and Brown 1997). BDNF elevates malonyl-CoA independently of AMPK; however, the effect of BDNF on muscle cell fatty acid oxidation is AMPK dependent (Matthews, Astrom et al. 2009). Skeletal muscle has a high nutritive demand, and poor intramuscular fatty acid metabolism may contribute to obesity (Houmard 2008) and insulin resistance (Hulver and Dohm 2004; Savage, Petersen et al. 2007). The finding that BDNF increases ACCβ phosphorylation and fatty acid oxidation may be one of the ways in which peripheral BDNF increases insulin sensitivity and weight loss. Muscle derived BDNF is not released into circulation (Matthews, Astrom et al. 2009), and although exercise is known to increase circulating levels of BDNF (Ferris, Williams et al. 2007), the source of this increase is unlikely from muscle cells (Matthews, Astrom et al. 2009).

In the liver BDNF contributes to the development of hyperglycemia, hyperinsulinemia, elevated serum cholesterol, and triglycerides associated with eating a HFD (Teillon, Calderon et al. 2010). When fed a HFD, liver specific BDNF knockout mice have elevated levels of peroxisome proliferator-activated receptor alpha (PPARα) and fibroblast growth factor 21 (Fgf21) compared with wild type mice (Teillon, Calderon et al. 2010). Fgf21 is a downstream target of PPARα, which is important for hepatic lipid oxidation, and insulin sensitivity (Kharitonenkov, Shiyanova et al. 2005; Badman, Pissios et al. 2007; Xu, Stanislaus et al. 2009). Fgf21 dose dependently reduces body weight and

adiposity and reduces the expression of a variety of genes involved in fatty acid and triglyceride synthesis (Xu, Stanislaus et al. 2009). The protection against HFD-induced hyperglycemia and hyperinsulinemia observed in liver specific knock-outs is inconsistent with previous research which reported improvements in liver histology after subcutaneous BDNF treatment (Yamanaka, Itakura et al. 2007). Thus, the improved liver histology viewed in these cases is likely secondary to other peripheral effects of BDNF.

Mature BDNF (~13.6 kDa), which is less than half the size of pro-BDNF (~32kDa), does not cross the blood brain barrier (Pardridge, Kang et al. 1994), and therefore studies reporting physiological effects of subcutaneous injections of BDNF may be reflective of peripheral actions. Subcutaneous BDNF enhances glucose utilization in muscle and BAT of db/db mice, but not normoglycemic animals (Yamanaka, Tsuchida et al. 2007). Additionally, BDNF restores levels of insulin secreting granules in beta cells and maintains their histologic cellular organization in db/db mice, even though there is no trkB receptor in pancreatic islets (Yamanaka, Itakura et al. 2006). Subcutaneous injections of BDNF reduced body weight, fat pad weight, and liver weight in db/db mice compared with pair fed TZD-treated db/db mice (Yamanaka, Itakura et al. 2007). A single injection of BDNF has a prolonged hypoglycemic effect, which is not attributable to reductions in food intake alone (Yamanaka, Itakura et al. 2008). Taken together, this indicates that BDNF may act peripherally to normalize blood glucose in hyperglycemic rodents, or in those no longer sensitive to leptin, possibly by enhancing muscle utilization or via the stimulation of BAT.

While both central and peripheral activation of the trkB receptor reduces food intake and obesity in rodents, this effect is not conserved across all species. In monkeys, the response of peripheral trkB activation is orexigenic and obesity promoting, while the central activation parallels the anorectic effects observed in mice (Lin, Tsao et al. 2008). The involvement of BDNF in energy homeostasis in

humans is difficult to study, and is usually limited to correlations between serum or plasma BDNF and body weight or adiposity. Serum BDNF levels are lower in human type-2 diabetic patients (Fujinami, Ohta et al. 2008), anorexia nervosa (AN) (Nakazato, Tchanturia et al. 2009; Saito, Watanabe et al. 2009) and in extremely overweight children (El-Gharbawy, Adler-Wailes et al. 2006), but not in bulimia nervosa (BN) (Saito, Watanabe et al. 2009), recovered AN (Nakazato, Tchanturia et al. 2009), or healthy controls (Nakazato, Tchanturia et al. 2009; Saito, Watanabe et al. 2009). Thus the relationship between serum BDNF and BMI is not clear. In one study there was a significant positive relationship between the two (Saito, Watanabe et al. 2009) and in another, serum BDNF negatively correlated with both BMI and body fat (El-Gharbawy, Adler-Wailes et al. 2006). It is likely that serum BDNF reflects the amount stored in platelets which is released during the clotting process (Fujimura, Altar et al. 2002; Mercader, Fernandez-Aranda et al. 2007). This level is not acutely altered by food intake (El-Gharbawy, Adler-Wailes et al. 2006). Plasma BDNF, on the other hand, likely has many sources (Braun, Lommatzsch et al. 1999; Kerschensteiner, Gallmeier et al. 1999; Nakahashi, Fujimura et al. 2000; Gielen, Khademi et al. 2003)(Braun A 1999, Nakahashi 2000, Gielen 2003, Kerschensteiner 1999). Plasma BDNF is higher in obese women, but these levels are significantly dropped after bariatric surgery (Merhi, Minkoff et al. 2009). Conversely, Mercader *et al.* observed no correlation between plasma BDNF and BMI in clinical subgroups of eating disorder patients (Mercader, Fernandez-Aranda et al. 2007). Krabbe *et al.* report that plasma levels of BDNF are decreased in human type 2 diabetics, independent of obesity (Krabbe, Nielsen et al. 2007). By sampling from the internal jugular vein and comparing plasma BDNF from arterial and venous samples, Krabbe *et al.* demonstrated that cerebral output of BDNF is reflected in the circulation. They observed that plasma BDNF levels are directly, inversely,

related to fasting plasma glucose levels, and that BDNF output from the brain is inhibited when blood glucose levels are elevated (Krabbe, Nielsen et al. 2007).

BDNF and neuronal plasticity

Neuronal plasticity is defined as an experience dependent change in synaptic strength (Bliss and Collingridge 1993). A well-studied example of this is long-term potentiation (LTP), which has particularly been associated with neurons of the hippocampus. LTP is the activity dependent strengthening of a synapse, which is typically induced by high frequency stimulation of excitatory input. Many studies have identified the importance of BDNF in the development of LTP (Korte, Carroll et al. 1995; Figurov, Pozzo-Miller et al. 1996; Patterson, Abel et al. 1996; Kang, Welcher et al. 1997; Xu, Gottschalk et al. 2000; Zakharenko, Patterson et al. 2003) and also in the development from LTP to long-term memories (LTM) (Alonso, Vianna et al. 2002; Tominaga-Yoshino, Kondo et al. 2002; Tominaga-Yoshino, Urakubo et al. 2008). BDNF is secreted in response to a high frequency stimulation, and is dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels or NMDA receptors (Hartmann, Heumann et al. 2001; Balkowiec and Katz 2002; Aicardi, Argilli et al. 2004). BDNF can bind trkB receptors on either side of the synapse (Drake, Milner et al. 1999). In the hippocampus it functions to facilitate the pre-synaptic release of excitatory neurotransmitters (Boulanger and Poo 1999), as well as post-synaptic AMPA receptor insertion (Li and Keifer 2008; Li and Keifer 2009) and dendritic spine maintenance (Chakravarthy, Saiepour et al. 2006; Danzer, Kotloski et al. 2008; Tanaka, Horiike et al. 2008). In contrast to LTP promotion by mature BDNF, recent evidence indicates a potential role for pro-BDNF in facilitating long-term depression (LTD) through activation of $p75^{NTR}$ receptor (Fahnestock, Michalski et al. 2001) (Lu 2003) (Woo, Teng et al. 2005).

Factors related to energy balance have been described to affect hippocampal LTP. When fed a diet high in both fat and sucrose (HFS), rats were less capable in a special learning capacity task than rats fed standard chow (Wu, Molteni et al. 2003). There was no sign of neuronal degeneration in the HFS fed rats. Additionally, rats with the lowest hippocampal BDNF, who also had lower levels of CREB and the vesicle associated synapsin I (Jovanovic, Benfenati et al. 1996), had the lowest learning capacity (Wu, Molteni et al. 2003). A HFD also has a negative impact on hippocampal LTP and plasticity (Porter, Kerr et al. 2010), and some studies suggest that this impairment is related to BDNF. A diet high in saturated fat and refined sugars significantly reduced hippocampal BDNF compared with rats fed standard chow, which was accompanied by impaired LTP and poor performance on the Morris water maze, a spatial learning task (Molteni, Barnard et al. 2002). A diet high in saturated fat reduced BDNF in both the ventral hippocampus and medial prefrontal cortex with associated impairment in reversal learning (Kanoski, Meisel et al. 2007). Taken together, these data suggest that a HFD may affect synaptic plasticity in rat hippocampal neurons. Conversely, caloric restriction (Fontan-Lozano, Saez-Cassanelli et al. 2007) and exercise (Stranahan, Khalil et al. 2006) enhance LTP and are associated with increased BDNF (Neeper, Gomez-Pinilla et al. 1996; Lee, Seroogy et al. 2002). Hippocampal BDNF levels are increased with running (Cotman, Berchtold et al. 2007), and running protects against stress related down-regulation of BDNF (Adlard and Cotman 2004).

Although the role of BDNF in neuronal plasticity has been well-studied in the hippocampus, the possibility that BDNF contributes to plasticity of hypothalamic neurons related to energy balance is less well-studied. The molecular mechanisms by which BDNF acts in the hypothalamus to affect energy homeostasis have not been characterized. However, an example of BDNF-dependent hypothalamic plasticity has been recently described in thermal

sensation and temperature control development (Katz and Meiri 2006). Several plasticity-related genes (including BDNF) were reported to be differentially expressed in high and low body weight chickens during development, suggesting different inherent capacities of these animals to adapt appetite circuitry (Ka, Lindberg et al. 2009). We have recently observed reductions in feeding behavior in rats after BDNF administration into the VMN and PVN. We observed this feeding inhibition between 4-24 and 24-48 hours post injection in both sites; however we did not observe an effect of BDNF during the first 4 hours (Wang, Bomberg et al. 2007; Wang, Bomberg et al. 2007). The possibility that BDNF acts to suppress food intake via a plasticity related mechanism in the hypothalamus is thus worth investigating. Neuronal activity in the PVN is modulated by excitatory glutamatergic signaling as well as other excitatory and inhibitory neurotransmitters. Recently NMDA receptor-dependent plasticity in the PVN has been described to reduce excitatory glutamatergic signaling in spontaneously hypertensive rats (Li, Yang et al. 2008), and the NMDA receptor-dependent plasticity was induced environmentally by chronic intermittent hypoxia (Coleman, Wang et al. 2010). We have observed that a single injection of BDNF in the PVN dose dependently suppresses feeding for up to 48 hours (Wang, Bomberg et al. 2007). The duration of the effect of a single injection of BDNF into the PVN, as well as characterization of plasticity mechanisms in the PVN, makes neural plasticity seem a candidate mechanism for the observed anorectic response.

BDNF and neurogenesis

The adult central nervous system contains neuronal stem cells capable of generating new neurons (Altman 1962) and several progenitor cells in certain brain regions have been identified (Temple 2001; Gould and Gross 2002). Neurogenesis has been well-studied in the subventricular zone of the lateral

ventricles, and the subgranular zone of the hippocampal formation (Gage, Kempermann et al. 1998). In the hippocampus, Nakatomi *et al.* observed the regeneration of CA1 pyramidal neurons of the adult rodent brain following ischemic degeneration, which was facilitated by the icv infusion of two different growth factors (FGF-2 and EGF). Novel to this study was the observation that growth factors signal the recruitment of progenitors from areas near the hippocampus to facilitate neurogenesis in areas where no progenitors are available. Thus extensive neurogenesis is possible in brain sites not containing stem cells (Nakatomi, Kuriu et al. 2002). Mitosis of progenitor cells lasts about 24 hours for the rat (Cameron and McKay 2001), and 14 hours in the mouse (Mandyam, Harburg et al. 2007), after which it takes three to four weeks for new neurons to mature and fully integrate into the circuitry (van Praag, Schinder et al. 2002). Thus any effect of BDNF on neurogenesis would likely be observable more long-term.

Energy balance has been described to affect BDNF and neurogenesis in the hippocampus. Dietary restriction (DR) increases neurogenesis in the adult mouse hippocampus, an effect associated with elevated BDNF expression, yet this effect is absent in BDNF heterozygous mice (Lee, Seroogy et al. 2002). However, DR normalizes BDNF in the hippocampus, striatum, and cerebral cortex of Huntington mutant mice, in whom BDNF expression is decreased, such that it is equivalent to the expression in wild type *ad libitum* fed mice, and reverses obesity, abnormal locomotor activity, and hyperphagia (Duan, Guo et al. 2003). Conversely, a diet high in saturated fat decreases BDNF and compromises cognitive performance (Molteni, Barnard et al. 2002). In mice susceptible to diet induced obesity (DIO), a HFD decreases BDNF and trkB mRNA in the hippocampus compared with mice resistant to diet induced obesity (Yu, Wang et al. 2009). Exposure to enriched environment and exercise elevates BDNF in the hippocampus, and improves learning (Olson, Eadie et al. 2006).

Conversely, it has been observed in rats that the consumption of a HFD, particularly one high in saturated fat, decreases BDNF and adult hippocampal neurogenesis (Park, Park et al. 2010). Park *et al.* observed that the prevention of neurogenesis was associated with increased levels of malondialdehyde (MDA) in the hippocampus, which is an indicator of lipid peroxidation, and they found a direct effect of MDA administration on inhibiting neurogenesis (Park, Park et al. 2010). A HFD fed to dams is associated with obesity and hyperlipidemia in offspring. These offspring have impaired hippocampal neurogenesis which parallels with the degree of neuronal impairment observed when treating cells in vitro with MDA (Tozuka, Wada et al. 2009). Tozuka *et al.* observed that a maternal HF diet caused reductions in hippocampal BDNF and impaired dendritic arborization of hippocampal neurons in pups (Tozuka, Kumon et al. 2010).

Neurogenesis in the hypothalamus is less well characterized. The effect of a maternal HFD on hypothalamic neurogenesis in pups has recently been investigated, where HFD increased neurogenesis of neurons expressing orexigenic peptides galanin, enkephalin and dynorphin in the PVN and orexin and melanin-concentrating hormone in the perifornical lateral hypothalamus (Chang, Gaysinskaya et al. 2008). These hypothalamic changes were associated with increased body weight, leptin, insulin, dietary fat preference, triglycerides, and galanin expression in the PVN (Chang, Gaysinskaya et al. 2008). Thus it appears that energy balance affects neurogenesis, however a compelling question that remains to be addressed is whether neurogenesis also affects energy balance?

Neuronal stem cells have been identified in the hypothalamus, and hypothalamic neurogenesis has been described to occur at a low rate (Kokoeva, Yin et al. 2007). Ciliary neurotrophic factor (CNTF) is similar to BDNF in that it promotes neuronal survival (Sendtner, Schmalbruch et al. 1992; Larkfors, Lindsay et al. 1994) and the maintenance of neuronal stem cells (Shimazaki,

Shingo et al. 2001), and activates signaling cascades in the hypothalamus involved in feeding and energy homeostasis (Bjorbaek, Elmquist et al. 1999; Lambert, Anderson et al. 2001). Kokoeva *et al*. observed an increase in hypothalamic neurogenesis in several hypothalamic feeding centers, particularly the median eminence-Arc, in CNTF-treated mice fed a HFD compared with non-CNTF treated controls. Furthermore, the CNTF-treated mice were resistant to weight gain on the HFD, which persisted for over a week after the cessation of CNTF treatment. The anti-mitotic agent cytosine-B-D-arabinofuranoside prevented the inhibition of weight gain after the cessation of CNTF treatment, but not during, indicating neurogenesis is partially responsible for the sustained anti-obesigenic effect of CNTF (Kokoeva, Yin et al. 2005). Additionally, the new neurons expressed proteins involved in energy balance such as POMC, neuropeptide Y, and phosphorylated STAT3, an indicator of leptin signaling (Kokoeva, Yin et al. 2005). That CNTF is capable of inducing hypothalamic neurogenesis, and that this neurogenesis has an anti-obesigenic effect is a demonstration that hypothalamic neurogenesis can, in fact, play an important role in regulating energy metabolism.

Using BrdU, Pencea *et al*. were the first to observe that BDNF is capable of inducing hypothalamic neurogenesis after continuous icv administration of BDNF for 12 days (Pencea, Bingaman et al. 2001). Neurogenesis triggered by BDNF correlates with levels of trkB expression, but this trkB receptor is not directly integrated into new neurons. Thus hypothalamic regions that contained high levels of the trkB receptor (e.g. the PVN) had greater amounts of BrdU cells than areas with less trkB expression, even if those areas were located closer to the site of BDNF infusion (Pencea, Bingaman et al. 2001). Recently, Kumar *et al.* used BrdU to measure neurogenesis in response to dietary restriction (DR) during a model of cytotoxic injury. Alternate day DR was associated with increased BDNF and neurogenesis in several brain regions, including the median

eminence-Arc of the hypothalamus (Kumar, Parkash et al. 2009). The type of new neurons generated in the Arc with dietary restriction remains to be elucidated as well as precisely how they might affect energy balance.

Neuroprotection and survival

During development, neurotrophic factors are critical to survival because they inhibit apoptosis of developing neurons (Wyllie, Kerr et al. 1980). BDNF is neuroprotective in the hippocampus, particularly against ischemic damage (Beck, Lindholm et al. 1994; Kokaia, Nawa et al. 1996; Larsson, Nanobashvili et al. 1999). Neuronal apoptosis, or programmed cell death, is characterized by the activation of caspases, specifically caspase 9 which acts upstream of caspase 3 (Thornberry and Lazebnik 1998). BDNF reduces glutamate-induced apoptotic cell death upstream of the activation of caspase-3-like enzymes and increases expression of the antiapoptotic protein B-cell lymphoma 2 (Bcl-2) (Almeida, Manadas et al. 2005). Additional studies have shown BDNF induces increases in Bcl-2 (Peng, Chiou et al. 2008; Assuncao, Santos-Marques et al. 2010; Kitazawa, Numakawa et al. 2010). It is important to note, however, that BDNF is not always protective. To some cultured neurons of the hippocampus and cerebrocortex BDNF is toxic (Friedman 2000). While activation of trkB receptors increases LTP and neuronal survival, activation of $p75^{NTR}$ can lead to apoptosis (Barrett 2000), and LTD (Woo, Teng et al. 2005).

Oxidative stress (Jones 2006) often leads to a loss of cell function, apoptosis or necrosis (reviewed in Azad, 2010 (Azad, Iyer et al. 2010)). BDNF has been implicated in being protective against oxidative stress by preventing the accumulation of peroxides and increasing antioxidant enzymes in hippocampal neurons (Mattson, Lovell et al. 1995). An abundant oxidative stress marker is 4-hydroxynonenal (HNE), which is generated through peroxidation of omega 6-polyunsaturated fatty acids (Benedetti, Comporti et al. 1980; Esterbauer, Schaur

et al. 1991). HNE is highly diffusible, and may contribute to oxidative stress far from a site of injury (Esterbauer, Schaur et al. 1991). Local application of BDNF on the dorsal hemisected spinal chord in rats resulted in reduced lipid peroxidation, as shown by decreased HNE-immunoreactive staining (Joosten and Houweling 2004). This effect was observed within 48 hours of BDNF application and was associated with a reduction in activated microglial cells, which may have contributed to decreased oxidative stress (Joosten and Houweling 2004).

In brain regions exhibiting neuronal loss, as is associated with Huntington's disease (Ferrer, Blanco et al. 2000) and Alzheimer's disease (Hock, Heese et al. 2000), BDNF levels are low, which contributes to the neuronal degeneration associated with these diseases (Hock, Heese et al. 2000). Tg2576 mice are a widely used model of Alzheimer's disease. They develop amyloid plaques and declining cognitive function at 6 months old (Westerman, Cooper-Blacketer et al. 2002). Kohjima *et al.* observed Tg2576 mice fed a HFD developed obesity and insulin resistance due to hyperphagia, and that the abnormal feeding behavior was associated with increased amyloid plaque formation and decreased hypothalamic BDNF (Kohjima, Sun et al. 2010). Oxidative stress is present early in the pathogenesis of Alzheimer's disease (Keller, Schmitt et al. 2005; Ringman, Younkin et al. 2008). Vitamin E is a known antioxidant, which can prevent the development of oxidative stress. Vitamin E supplementation ameliorates HFD-induced reductions in BDNF, suggesting that BDNF levels may be altered in response to oxidative stress (Wu, Ying et al. 2004). Several studies have identified that there is a relationship between a HFD and impairments in cognitive performance, particularly in rats fed a diet high in saturated fats (Greenwood and Winocur 1996; Winocur and Greenwood 1999; Greenwood and Winocur 2005; Winocur and Greenwood 2005). In addition to affecting cognitive function, a HFD induces apoptosis of hypothalamic neurons,

such as POMC neurons (Moraes, Coope et al. 2009). Thus, the neuroprotective effect of BDNF may have a role in the central regulation of energy metabolism; however further studies are needed to define the role of BDNF in hypothalamic neuroprotection and how it relates to energy balance.

Factors affecting expression of hypothalamic BDNF

Hypothalamic BDNF and trkB content are affected by age. In rats raised in standard laboratory conditions, the mature form of hypothalamic BDNF peaks at about 1 week post-natally in rats, remains elevated for the first month of life, and declines with age (Silhol, Bonnichon et al. 2005). Additionally, declining levels of trkB receptor begin in rats at around two months, with extreme reductions observed by 22 months (Silhol, Bonnichon et al. 2005). It is possible that environmental factors could prevent age-related decline in BDNF. Recently Cao *et al.* reported BDNF expression is increased in mice housed in an enriched environment. The environmental enrichment included a large open space, toys, and a running wheel (Cao, Liu et al. 2010). Earlier studies have shown that in this type of complex environment, animals exhibit improved learning and memory and increased neurogenesis (Cao, Jiao et al. 2004; During and Cao 2006). Additionally, mice living in an enriched environment remain leaner than mice in standard housing, an effect associated with consistently elevated BDNF expression in the Arc (Cao *et al*., unpublished data referenced in (Cao, Lin et al. 2009)). Elevated BDNF in the hypothalamus associated with environmental enrichment decreased leptin levels, which inhibited cancer tumor growth in several models of cancer. The metabolic profiles of mice in an enriched environment were mimicked when BDNF was over expressed in the hypothalamus of animals living in standard housing using a recombinant Adeno-Associated Virus (rAAV) vector (Cao, Liu et al. 2010).

Four weeks of running increased slightly, but not significantly, expression of BDNF in the Arc, whereas an enriched environment significantly increased Arc BDNF expression after only two weeks. While both the running mice and the enriched environment mice had similar decreases in body weight, the groups differed in metabolic gene expression and only the enriched environment group had a corresponding decrease in leptin and tumor growth (Cao, Liu et al. 2010). The study by Cao *et al*. opens the door to many questions about the relationship between environment and metabolism, more specifically, how does environmental enrichment impact food intake and energy expenditure to contribute to obesity resistance? What constitutes an enriched environment? In another model of environmental enrichment, Angelucci *et al.* report that music increased BDNF in the mouse hypothalamus (Angelucci, Ricci et al. 2007).

Acute immobilization stress causes rapid increases in hypothalamic BDNF (Rage, Givalois et al. 2002; Naert, Ixart et al. 2006). These increases are accompanied by decreased body weight and increased locomotor activity, as well as activation of the HPA (Naert, Ixart et al. 2006). Conversely, neonatal stress induced by separating rat pups from their dams for 180 minutes per day is associated with elevations in hippocampal BDNF protein expression, which is likely exerting a protective effect on existing neurons there (Greisen, Altar et al. 2005).

Kohjima *et al*. report that 16 weeks of a HF diet, which was sufficient to induce insulin resistance, obesity, and amyloid plaques in Tg2567 mice, decreased hypothalamic BDNF and led to elevated feeding behavior (Kohjima, Sun et al. 2010). In the VMN specifically, BDNF, but not trkB mRNA, is lower in DIO mice compared with DRO, suggesting that the DIO phenotype may in part be mediated through low BDNF expression in this important feeding center (Yu, Wang et al. 2009). No differences in BDNF expression were observed between the two phenotypes in the arcuate nucleus, the DMN or the PVN (Yu, Wang et al.

2009). However, Sprague-Dawley rats fed either a high-energy diet (HE) or high energy plus Ensure liquid diet (HE + E) expressed increased trkB receptor, but not BDNF, in the VMN (Archer, Rayner et al. 2005). Both groups decreased food intake and body weight when switching from their respective diets to standard chow, and in both cases this was accompanied by decreased BDNF. The HE, but not the HE+E group also had decreased trkB expression upon switching to standard chow (Archer, Rayner et al. 2005). Differences in trkB mRNA are difficult to interpret, because not all forms of trkB are active and some might serve to inhibit trkB signaling. We have recently demonstrated that chronic administration of BDNF into the PVN reduced HFD-induced obesity, and that animals with greater body fat were more responsive to the effect of added BDNF (Wang, Godar et al. 2010). This is in line with recent evidence which suggests that animals resistant to HFD-induced obesity maintain higher basal levels of BDNF and trkB in the hypothalamic VMN compared with animals susceptible to diet induced obesity (Yu, Wang et al. 2009). Moreover, VMN BDNF levels were further reduced in DIO mice on a HF diet, and negatively correlated with plasma glucose and positively correlated with plasma adiponectin (Yu, Wang et al. 2009). Adiponectin is an adipokine associated with increased insulin sensitivity and reduced appetite (Berg, Combs et al. 2001). This suggests that BDNF and trkB expression in the hypothalamus might play a role in determining susceptibility to obesity (Yu, Wang et al. 2009). The anti-obesity effect of BDNF in the PVN was related to a significant reduction in energy intake (Wang, Godar et al. 2010), and previously we have observed that BDNF in the PVN increased energy expenditure and resting metabolic rate (Wang, Bomberg et al. 2007).

Perspectives and Significance

BDNF deficiency is associated with increased weight in mice and humans, and BDNF administration in the hypothalamus can reduce food intake and

increase energy expenditure, leading to lighter animals. The two critical hypothalamic sites are the PVN and VMN, but other brain and peripheral sites may also play a role. There is strong evidence that BDNF in the hippocampus is involved in neural plasticity and neurogenesis in adult animals, but whether hypothalamic BDNF exerts its energy balance effects through plasticity and neurogenesis has not yet been determined. There is evidence that the BDNF-induced negative energy balance persists long after BDNF administration, which would be compatible with plastic mechanisms. Much more investigation is needed concerning whether hypothalamic BDNF mediates energy balance, in part, via plasticity and/or neurogenesis, and whether hippocampal and hypothalamic BDNF are influenced by environment and energy states.

Chapter 4

Exercise reduces energy balance by reducing feeding and elevates BDNF in the hypothalamic PVN

Introduction

Obesity affects has deleterious effects on many aspects of health and well-being. Despite considerable public health efforts to combat obesity, in 2011-2012 the prevalence of obesity was reportedly nearly 35% of adults and 17% of children, similar to obesity prevalence in 2003 (Ogden, Carroll et al. 2014). The etiology of obesity is physiologically individualistic and multifaceted, but its immediate cause is a chronic energy imbalance, where more calories are consumed than expended. Exercise is recommended as a method of controlling weight, as it increases overall energy expenditure. However, recent studies suggest exercise may cause compensatory reductions in non-exercise activity thermogenesis, and therefore may not be a good strategy for effectively promoting weight loss (Colley, Hills et al. 2010). Nevertheless, epidemiological evidence shows an inverse correlation between physical activity and body mass index among obese adults (Hemmingsson and Ekelund 2007) and an inverse association between energy expended in high intensity exercise and overweight/obesity (Tucker and Peterson 2003; Bernstein, Costanza et al. 2004).

As early as 1970 it was proposed that exercise can prevent obesity not only by increasing energy expenditure, but also by preventing excessive feeding (Baile, Zinn et al. 1970). However, this aspect of exercise has not been widely studied. Recent data suggests that exercise reduces meal size (Thivel, Isacco et al. 2012) (Bergouignan, Momken et al. 2010) and normalizes insensitive appetite control compared to sedentary controls (Martins, Truby et al. 2007). Additionally, exercise has been reported to improve satiety (King, Caudwell et al. 2009;

Martins, Kulseng et al. 2010) and increase post-prandial ratings of fullness (Rosenkilde, Reichkendler et al. 2013).

Though rodent studies have long reported an interesting paradox of exercise-induced hypophagia (Edholm, Fletcher et al. 1955; Stevenson, Box et al. 1966), the mechanisms are still not well known. There is evidence to suggest that in volitional running wheel exercise might affect plasticity of appetitive circuitry in rodents. For example, in juvenile rats running wheel exercise is associated with reduced adiposity and failure to increase intake of a palatable high-fat diet, despite the increased energy cost of running (Patterson, Dunn-Meynell et al. 2008). Remarkably, after six weeks of exercise, these animals refrained from overeating for seven weeks after running wheels were locked, while food restricted animals with comparable weight loss but without access to running ate significantly more of the high-fat diet when allowed ad libitum feeding (Patterson, Dunn-Meynell et al. 2008). Thus, while both food restriction and exercise promoted similar protection against weight gain, exercise benefits extended beyond the intervention time to protect against food increases and weight gain, whereas food restriction did not, suggesting that exercise may affect plasticity of appetitive mechanisms.

The effects of exercise in neuronal plasticity might be mediated by brain-derived neurotrophic factor (BDNF). BDNF is a member of the neurotrophin family of growth factors, with well defined roles in development of LTP (long term potentiation) (Korte, Carroll et al. 1995; Figurov, Pozzo-Miller et al. 1996; Patterson, Abel et al. 1996; Kang, Welcher et al. 1997; Xu, Gottschalk et al. 2000; Zakharenko, Patterson et al. 2003), neuronal survival (Grothe and Unsicker 1987; Hofer and Barde 1988; Kalcheim and Gendreau 1988) and neurogenesis (Alderson, Alterman et al. 1990; Knusel and Hefti 1991). Both BDNF and its receptor tropomyosin-related kinase B (trkB) (Klein, Nanduri et al. 1991) are present in pre-synaptic axon terminals and post-synaptic dendritic compartments, and are capable of bidirectional release of neurotransmitters and

activity (Tyler, Alonso et al. 2002). In rodents, central administration of exogenous BDNF promotes appetite suppression and weight loss (Pelleymounter, Cullen et al. 1995; Wang, Bomberg et al. 2007; Wang, Bomberg et al. 2007; Wang, Godar et al. 2010), increases locomotor activity (Naert, Ixart et al. 2006) and resting metabolic rate (Wang, Bomberg et al. 2007; Wang, Bomberg et al. 2010). In humans, a mutation in the trkB receptor causes severe obesity (Yeo, Connie Hung et al. 2004), suggesting that BDNF effects on energy homeostasis are conserved across species.

We investigated the contribution of exercise effects on feeding and the reduction of the progression of obesity in adult rats. We used both voluntary and forced exercise paradigms, and included two pair-fed groups to distinguish the effects of exercise on caloric intake to changes in body composition. The paraventricular nucleus (PVN) contains high levels of BDNF and trkB mRNA (Tapia-Arancibia, Rage et al. 2004). Furthermore, wheel running increases expression of the trkB receptor in the PVN (Cao, Choi et al. 2011). In the PVN, BDNF injections decrease 24-hour food intake, body weight gain and elevate resting metabolic rate and heat production (Wang, Bomberg et al. 2007). Therefore, we hypothesized that exercise increases expression of BDNF and trkB in PVN compared to sedentary controls. After eight weeks of exercise we did not observe increased PVN BDNF. However, at this time animals were no longer in negative energy balance. We then performed an acute exercise trial (five days) to measure BDNF while the animals were in negative energy balance. We report that exercise elevated PVN BDNF during the first week of the initiation of training.

Methods

Animals and exercise protocol

Adult male Sprague-Dawley rats (>1 y old) (Charles River, Wilmington, MA) were individually housed in cages and maintained on a 12:12-h light dark cycle (lights

on at 0400). Rooms were maintained at 21-22°C. Animals had ad libitum access to water and Research Diets control chow (D12450B). All protocols were approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Medical Center and University of Minnesota prior to experimentation.

General experimental protocols

Experiment 1

Animals were randomized into groups based of equal body composition and body weight. Animals were assigned to either running wheel (RW; n=8), treadmill (T; n=9), sedentary (S; n=9) or to one of two pair-fed groups where animals were sedentary and fed isocaloric amounts to either RW (PFRW; n=10) or T (PFT; n=8). Pair feeding was delivered twice a day, 1/3 of the daily amount was delivered at the onset of the light cycle and 2/3 was delivered one hour into the dark cycle. Food allocations were based on the average amount of food in grams/body weight eaten by the respective exercise group to whom the pair-fed animals were being calorically matched. Food intake and body weight were measured every 48 h and body composition measured once weekly using echoMRI-700 (Echo Medical Systems). The behavioral experiment lasted for eight weeks.

Experiment 2

Animals were randomized into one of three groups based of equal body composition and body weight: RW (n=7), S (n=6) or PFRW (n=6). The protocol was otherwise similar to Experiment 1, with the exception of the behavioral experiment being terminated and tissues collected after five days.

Exercise protocols

Animals were either singly housed in standard cages, or, where noted, were singly housed in standard cages with ad libitum access to running wheels (RW). For the RW group, running wheels, which animals could access at will,

were externally fixed to standard shoebox cages via a short tunnel. Running was monitored using Activity Wheel Monitoring Software (Lafayette Instrument, Lafayette, IN). Animals subjected to forced treadmill exercise (T) were acclimated gradually for 1 week using interval training, and performed treadmill exercise 5 days per week at a moderate pace (~15 m/min) for 45 min/day. The treadmill consists of a single belt containing 5 individual lanes with foot shock available at the end of the belt (Harvard Apparatus, Holliston, MA). Motivators were used to keep the animals running on the treadmill including a puff of air, foot shock (0.2 mA), or a wire bristle brush. Use of the brush, the air puff or the foot shock was animal dependent. Only when the rats were reluctant to run, these stimuli were used. The use of the foot shock was a rare event (< 2x per week) in order to minimize stress.

Body composition

An EchoMRI-700 (Houston, TX) was used to measure fat mass, lean mass, free water and total water in the rats. Each animal was weighed and then placed into a plastic holders based on their body weight with limited restraint. Then the holder was placed in the EchoMRI machine for scanning. Each scan took 1–2 minutes. Earlier, we have shown that the fat mass and lean mass measured with the MRI is consistent with those chemically analyzed, with an almost 1:1 correlation (Nixon, Zhang et al. 2010). Due to a technical failure of our Echo MRI machine, we were unable to obtain accurate body composition measurements at week eight of the study.

Immunofluorescence and unbiased stereology

Experiment 1

Animals were perfused transcardially using 4% paraformaldehyde. Brains were collected and post-fixed for 12 h in 4% paraformaldehyde, then transferred through a gradient to 30% sucrose and finally stored in cryoprotectant at -20C until ready for use. Brain tissue was transferred to cold PBS for 48 h before

sectioning to either 25 μm using a freezing stage microtome (American Optical Co. model 860, Buffalo, NY). Systematic random sampling was used for tissue collection and a total of six series were collected. Section collection began roughly at 1.8 mm posterior to bregma and ended at 2.12 mm posterior, according the Paxinos and Watson rat brain atlas (Paxinos and Watson 2007). Approximately 3-4 sections per animal were stained using the following procedure: free-floating sections were blocked using 5% normal donkey serum (NDS; Jackson Laboratories, West Grove, PA) in 0.01 M PBS with 0.3% Triton X-100. Tissue was then incubated in primary antibodies chicken anti-BDNF (Promega, Madison, WI; 1:100) and rabbit anti-trkB (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200) for 72 h at 4°C with gentle agitation. Sections were then washed in .01 M PBS and incubated in secondary antibodies: donkey anti-chicken Cy3 (Jackson Laboratories, West Grove, PA; 1:200) and donkey anti-rabbit Alexafluor 647 (Jackson Laboratories, West Grove, PA; 1:150) for two hours at 22°C in the dark. Sections were mounted onto slides and coverslipped using VECTASHIELD hardset mounting medium with DAPI (Vector Laboratories, Burlingame, CA). An image at 5x magnification was collected from each hemisphere containing the PVN using a Zeiss Axio Imager M2 (Gottingen, DE). The region of interest (ROI) was outlined and a random offset grid drawn onto the image using imageJ software (NIH, Bethesda, MD). At each grid crossing within the ROI an image was collected at 63x magnification. Optical sectioning was performed through 7 μm of the tissue using the Z stack function of Axiovision Software (Carl Zeiss Vision, version 4.8). Unbiased stereological methods were performed in order to count BDNF positive cells (West and Gundersen 1990). Briefly, images were uploaded into image J where cells were counted using an unbiased counting frame (40 x 40 μm). In order to avoid counting cells more than once, cells were counted only when “tops” of nuclei first came into focus. The estimated number of cells containing respective staining in each reference space (N) was calculated using the fractionator method (Gundersen 1986):

$$N = \Sigma Q^{-} * 1/ssf * 1/asf * 1/tsf$$

where ssf = the number of sections analyzed/total number of sections, asf = the area of the dissector frame/ area of the xy step in the random offset grid and tsf = the dissector height/section thickness.

Experiment 2

We obtained Stereoinvestigator software (Stereoinvestigator; MBF Bioscience) prior to initiation of Experiment 2. Brain tissues were fixed and post-fixed as described in Experiment 1 and transferred to 20% sucrose solution for a minimum of 72 hours before sectioning to 40 µm on a cryostat. Systematic random sampling was used for tissue collection and a total of 3 series were collected. Section collection began roughly at 1.8 mm posterior to bregma and ended at 2.12 mm posterior, according the Paxinos and Watson rat brain atlas (Paxinos and Watson 2007). Approximately 3-4 sections per animal were stained using the following procedure: for antigen retrieval, free-floating sections were heated to 80°C for 30 minutes in sodium citrate buffer (pH 8.0) and allowed to cool to room temperature before washing and blocking. Tissues were blocked and stained as described in Experiment 1. Sections were mounted using ProLong Gold antifade reagent (Cell Signaling Technology Inc, Beverly, MA). Stereoinvestigator was used for unbiased stereological quantification of BDNF. ROIs were drawn using anatomical landmarks for consistency. A guard distance of 1 µm from the top and bottom of each sampling site was excluded from counting. Sampling sites were determined using a random offset grid, as described in Experiment 1. The dissector size was (40 x 40 µm). The equation used for data analysis was the same as for Experiment 1.

Quantification of trkB immunoreactivity $trkB_{ir}$ in fibers

Fibers were quantified at 5x magnification using modified methods of Sathyanesan et al. (Sathyanesan, Ogura et al. 2012). Briefly, images were filtered using the feature J software plugin to select the Hessian-based filter in NIH image J (version 1.44). Pixel density was measured along 10 equidistant lines and number of fiber crossings was estimated using a threshold based on background pixel density. Data are presented as density of $trkB_{ir}$ in fibers:
(Σ (n peaks/ line length))/ n lines

Statistics: For comparison of exercise effects on cumulative food intake, we used two-factor analysis of variance (ANOVA) (exercise x time) followed Holm-Sidak's post-hoc analysis for multiple comparisons. Comparisons of body composition, cumulative feeding at four weeks, fiber density, and the number of BDNF positive cells was performed using one-way ANOVA followed by Holm-Sidak's post-hoc analysis for multiple comparisons. For the effect of exercise on body composition over time, we used two-factor ANOVA followed by Holm-Sidak's post-hoc analysis. Linear correlations were used to analyze the relationship between body composition and daily running distances. A two-tailed unpaired t-test was used to compare running wheel and treadmill average daily running distances. All statistics were performed using Graphpad Prism version 5.0 (Graphpad Software Inc.).

Results

There was a significant effect of exercise on cumulative food intake ($F_{2,506}$=64.3, p<0.0001). Running wheel exercise was associated with reduced feeding beginning at day 24, and treadmill exercise beginning at day 28 (p<0.05) **(Figure 4.1)**. In order to determine the effect of feeding alone on body composition changes with exercise, animals were pair-fed isocaloric diets to exercised animals, but were not exercised. After four weeks of the intervention,

exercise reduced cumulative food intake and body weights **(Figure 4.2)**. Similar to exercise, pair-feeding was associated with significantly reduced body weights (p<0.05), indicating that calorie intake contributed to exercise-induced weight loss (**Fig 4.2B**). Pair-feeding reduced body weights and fat/lean ratios, indicating that some of the body composition improvements conferred by exercise are a result of exercise-induced caloric deficits **(Fig. 4.2A, B)**. The effect of treatment on body weight change over time depended on the treatment group (interaction: treatment group x time: $F_{16, 156}$=9.7, p<0.0001) **(Figure 4.3A)**. Being sedentary caused weight gain throughout the eight-week study, whereas both running wheel and treadmill exercise protected against weight gain **(Fig. 4.3A)**. Pair-feeding also protected against weight gain, indicating that exercise-induced feeding reductions contributed to the protection against weight gain. By week eight, however, the caloric intake of animals who were exercising had increased such that pair fed animals had gained weight relative to baseline and animals calorically matched to treadmill runners were in positive energy balance **(Fig. 4.3A)**. There was a significant interaction (treatment group x time: $F_{12, 117}$=9.7, p<0.0001) for the effect of treatment group on fat/lean mass. Most striking were the positive improvements in fat/lean mass due to running wheel exercise (p<0.05) **(Fig. 4.3B)**. Treadmill exercise conferred similar benefits in fat/lean mass as pair-feeding, whose fat/lean mass was maintained at around baseline levels through week six. Changes in fat/lean mass were significantly higher in sedentary animals compared with all other groups at weeks four and six (p<0.05) **(Fig. 4.3B)**.

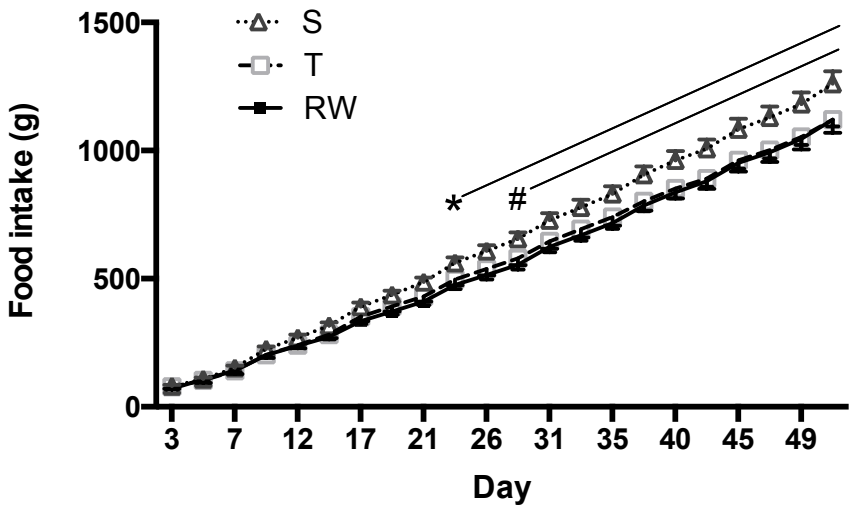

Figure 4.1 Exercise reduces feeding

Both voluntary running wheel (RW) and forced treadmill (T) exercise were associated in reduced cumulative food intake compared with sedentary controls (S) beginning at day 24 for RW (*p<0.05) and day 28 for T (#p<0.05); n=8-9.

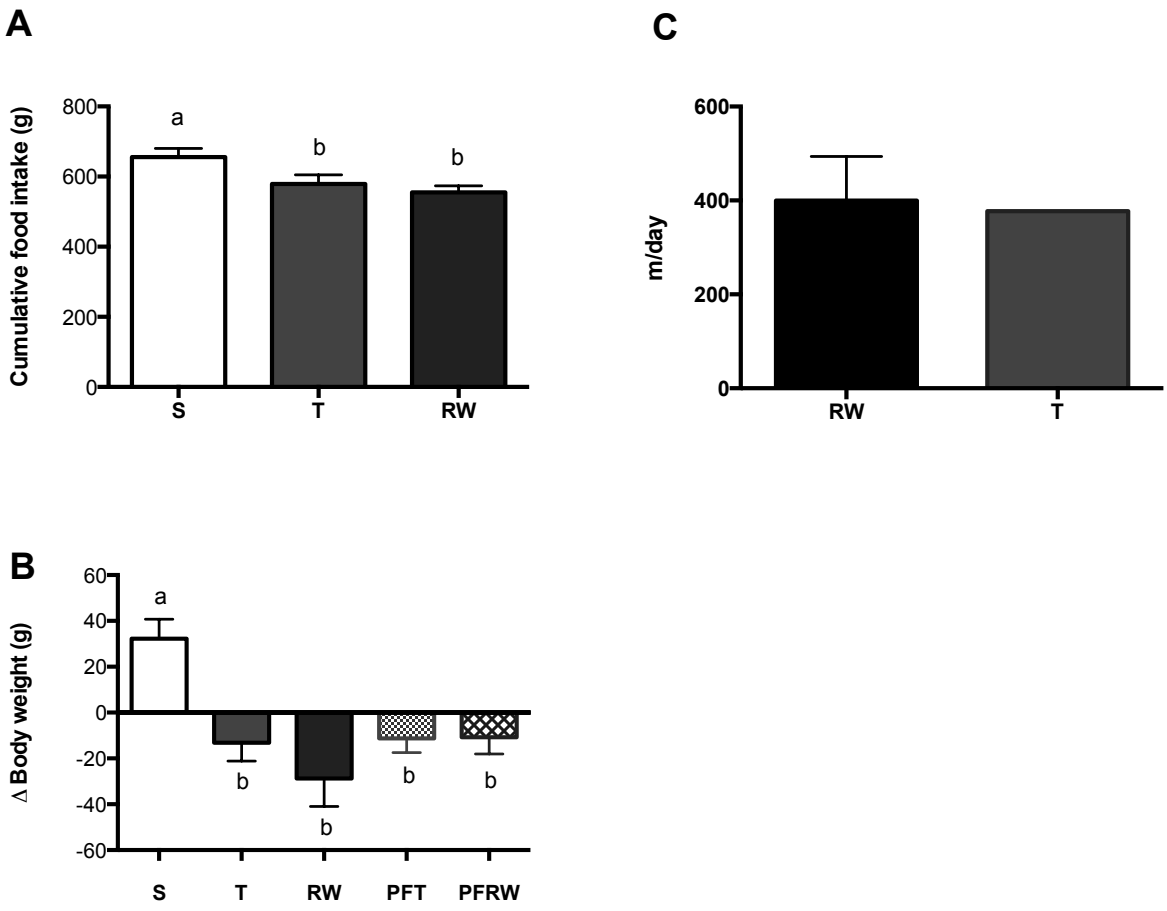

Figure 4.2 After four weeks of the intervention, reduced feeding contributed to weight loss during both voluntary and forced exercise.

Exercise reduced feeding (A) and body weight (B) in voluntary running wheel (RW), treadmill (T), pair-fed treadmill (PFT), pair-fed running wheel (PFRW) animals compared with sedentary (S) controls. At four weeks average daily running distances were not different between T and RW animals (C). Letters that differ from each other indicate significant differences ($p<0.05$; n=8-10).

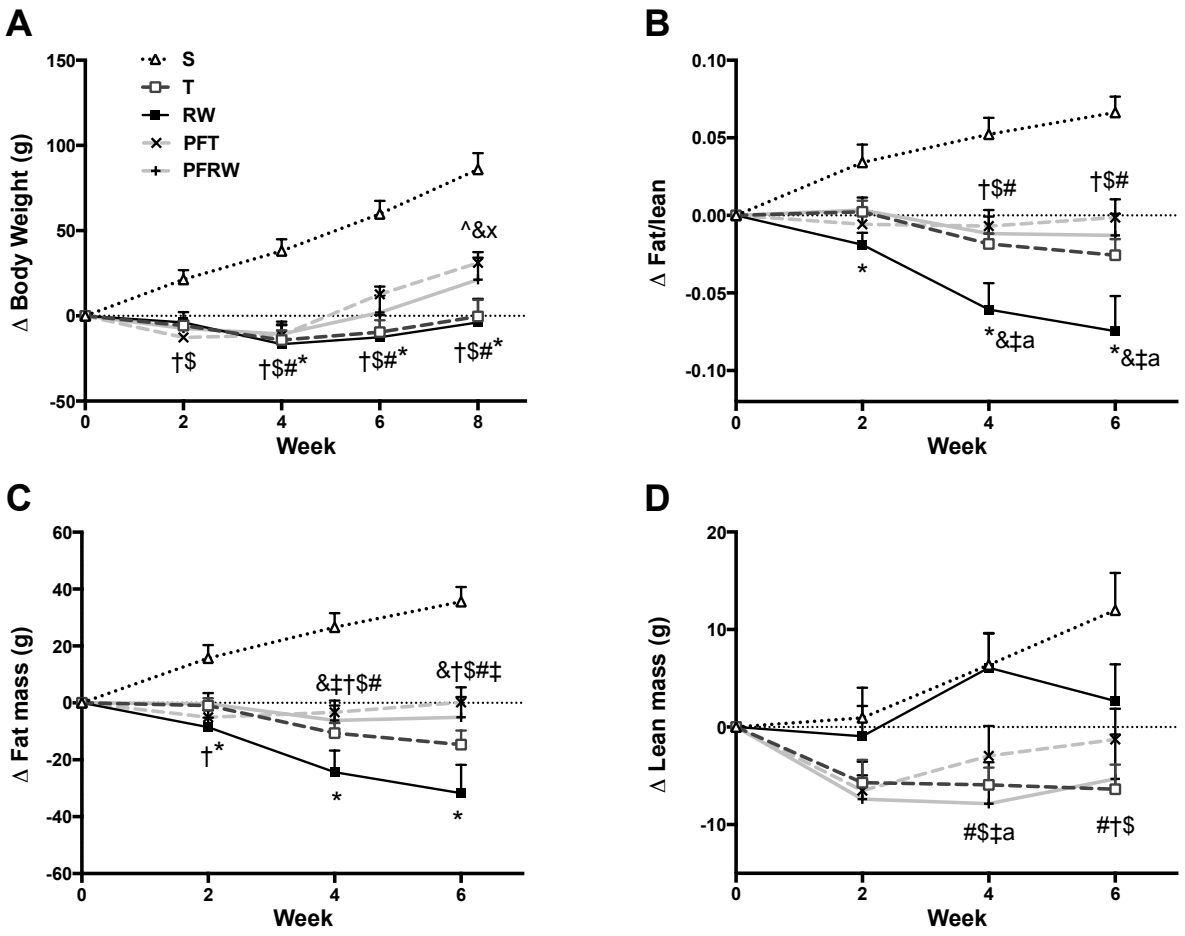

Figure 4.3 Time course of body composition changes due to either voluntary or forced exercise or pair feeding compared with sedentary controls.

Changes in body weight (A), fat/lean mass (B), fat (C) or lean mass (D) are all relative to baseline. Symbols indicate statistical significance (p<0.05, n=8-10) †S vs PFT, $^{\$}$S vs PFRW, $^{\#}$S vs T, *S vs RW, ^T vs PFT, $^{\&}$RW vs PFT, ‡RW vs PFRW, ^{a}T vs RW, ^{x}PFT vs baseline.

Changes in fat mass over time depended on treatment group (treatment group x time: $F_{12, 117}$=11.5, p<0.0001). Sedentary animals gained a significant amount of

fat mass compared with all other groups, who either lost fat or had no change in fat from baseline **(Fig. 4.3C)**. At weeks four and six, both exercise animals had significantly greater fat loss compared with sedentary and pair-fed animals, but there was no difference between T and RW groups ($p<0.05$). Changes in lean mass over time depended on treatment group (treatment group x time: $F_{12, 117}=3.3$, $p<0.0003$). By week four, running wheel exercise was associated with significantly greater gains in lean mass compared with treadmill exercise or pair-feeding ($p<0.05$) **(Fig. 4.3D)**, however these differences were no longer present at week six. By week six all groups had lost lean mass relative to baseline and to sedentary controls, who gained both lean and fat mass **(Fig 4.3C, D)**.

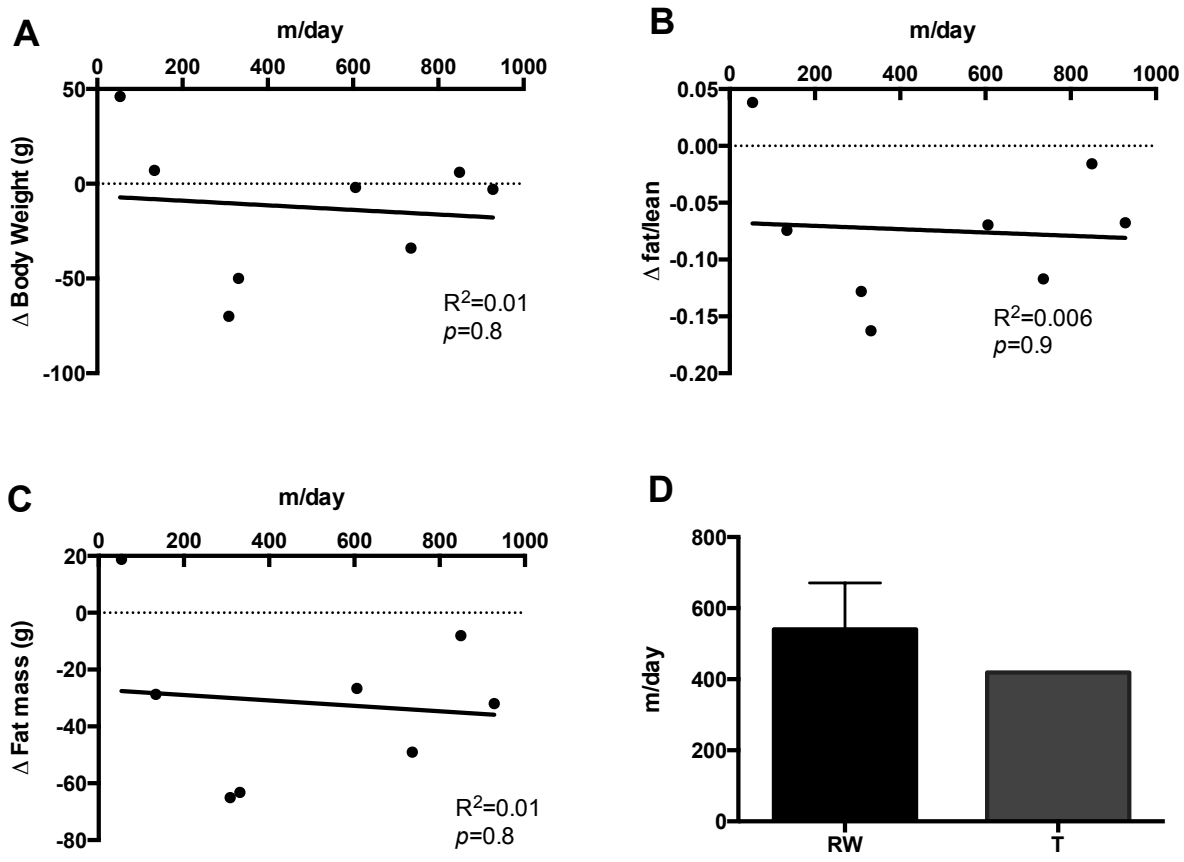

Figure 4.4 Running wheel distance is not correlated with changes in body weight or body composition.

After six weeks, daily distance on the running wheel was not correlated with body weight (A), fat/lean mass (B) or body fat (C) changes compared with baseline. These data suggest that exercise alone is not driving improvements in body composition observed when voluntary running is made available. At eight weeks, the average daily distances run by running wheel (RW) animals was not different from treadmill (T) animals, but tended to be higher. $p<0.05$, n=8 (A-C) and n=8-10 (D).

We examined whether running distance contributed to changes in body composition. **Figure 4** shows correlations between body composition

measurements and average daily running distances. There was no correlation between average daily distance run and body weight change (R^2=0.01, *p*=0.8 **(Fig. 4.4A)**, fat/lean mass change (R^2=0.006, *p*=0.9) **(Fig. 4.4B)** or fat mass change (R^2=0.01, *p*=0.8) **(Fig. 4.4C)** compared with baseline. Analysis at four-week time point also failed to show significant correlations (data not shown). These data suggest that exercise alone is not driving improvements in body composition observed when voluntary running is made available. Similar to week four, by week eight treadmill-exercised animals were running similar average daily distances to running wheel animals **(Fig. 4.4D)**. Taken together, these data suggest that the option for voluntary running is beneficial for improving body composition independently of the amount of running accomplished.

Since the most robust feeding and body composition changes were observed in animals in the running wheel group, we quantified the number of BDNF immunoreactive cells in the PVN of running wheel, pair fed running wheel and sedentary animals. At the eight-week time point we found no differences in PVN BDNF between sedentary, running wheel and pair-fed animals **(Figure 4.5)**.

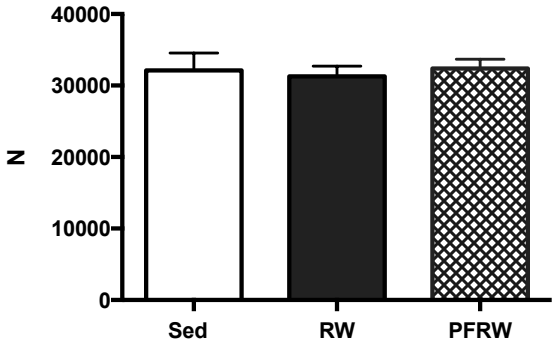

Figure 4.5 Eight weeks of running wheel access does not affect the number of brain derived neurotrophic factor positive cells in the hypothalamic paraventricular nucleus.

We found that most of the trkB expression in the PVN was present in fibers surrounding the PVN. Thus we hypothesized that all groups of exercised animals would have a higher density of trkB immunoreactive ($trkB_{ir}$) fibers compared to sedentary animals. We did find a significant effect of exercise on $trkB_{ir}$ fiber density ($F_{2, 37}$=6.7, p<0.003), however, $trkB_{ir}$ fiber density was significantly higher in treadmill compared with running wheel-exercised animals (p<0.05) **(Figure 4.6)**.

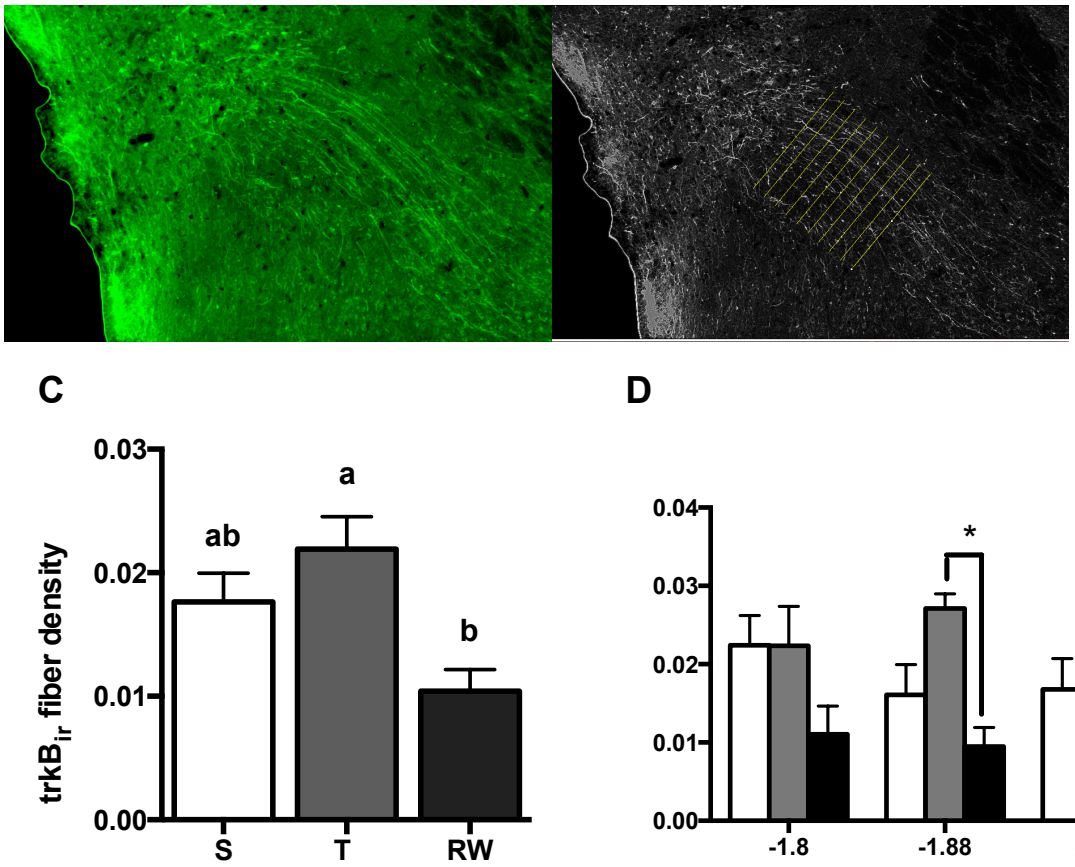

Figure 4.6 Running wheel exercise is associated with reduced trkB$_{ir}$ fiber density in regions surrounding the paraventricular nucleus (PVN).

TrkB fibers surrounding the PVN are shown in green (A). Methods for fiber quantification were adopted from Sathyanesan et al (2012)(Sathyanesan, Ogura et al. 2012). Images were filtered using a Hessian-based filter and 10 equidistant lines were drawn over the image (B). The number of fiber crossings was estimated using a threshold based on background pixel density. After eight weeks, there was a significant effect of exercise group on fiber density ($F_{2,\ 37}$=6.7, p<0.003) (C). Animals with running wheel access (RW) had a reduced number of trkB fibers surrounding the PVN compared with animals exercised on a treadmill (T). When broken down by anatomical location, there was one site where there was a significant effect of exercise on fiber density ($F_{2,\ 12}$=5.2, p<0.02)

was at 1.88 mm caudal to bregma according to Paxinos and Watson (Paxinos and Watson 2007).

These results suggested that BDNF is involved in exercise-induced plasticity changes in PVN during the initial stages of an exercise regime. We repeated the protocol from Experiment 1, including only the running wheel, sedentary and running wheel pair fed groups, because these animals had the greatest changes in body composition and the earliest divergence from sedentary animals in cumulative food intake **(Figure 4.7)**. Again we found that weight change over time (compared with baseline) depended on treatment group (exercise treatment x time) ($F_{8, 64}$=3.7, p<0.001) **(Figure 4.7B)**. By the fifth day post introduction of the running wheel, both the running wheel and pair-fed groups had significant reductions in body weight compared with sedentary controls (p<0.05) **(Figure 4.7A)**, suggesting that food intake was an important contributor to changes in energy balance. Food intake tended to be lower in exercised animals compared with sedentary controls, but the difference was not significant (p=0.08) **(Figure 4.7C, D)**. We perfused the animals on the fifth day and quantified the in number of BDNF immunoreactive cells in brain tissue. We found that running wheel exercise increased BDNF cell number whereas pair feeding did not **(Figure 4.8)**. These data suggest that BDNF may be elevated during the initial stages of running, but it is not caused by reduced feeding or energy balance, since pair-fed animals were also in negative energy balance but did not have elevated PVN BDNF.

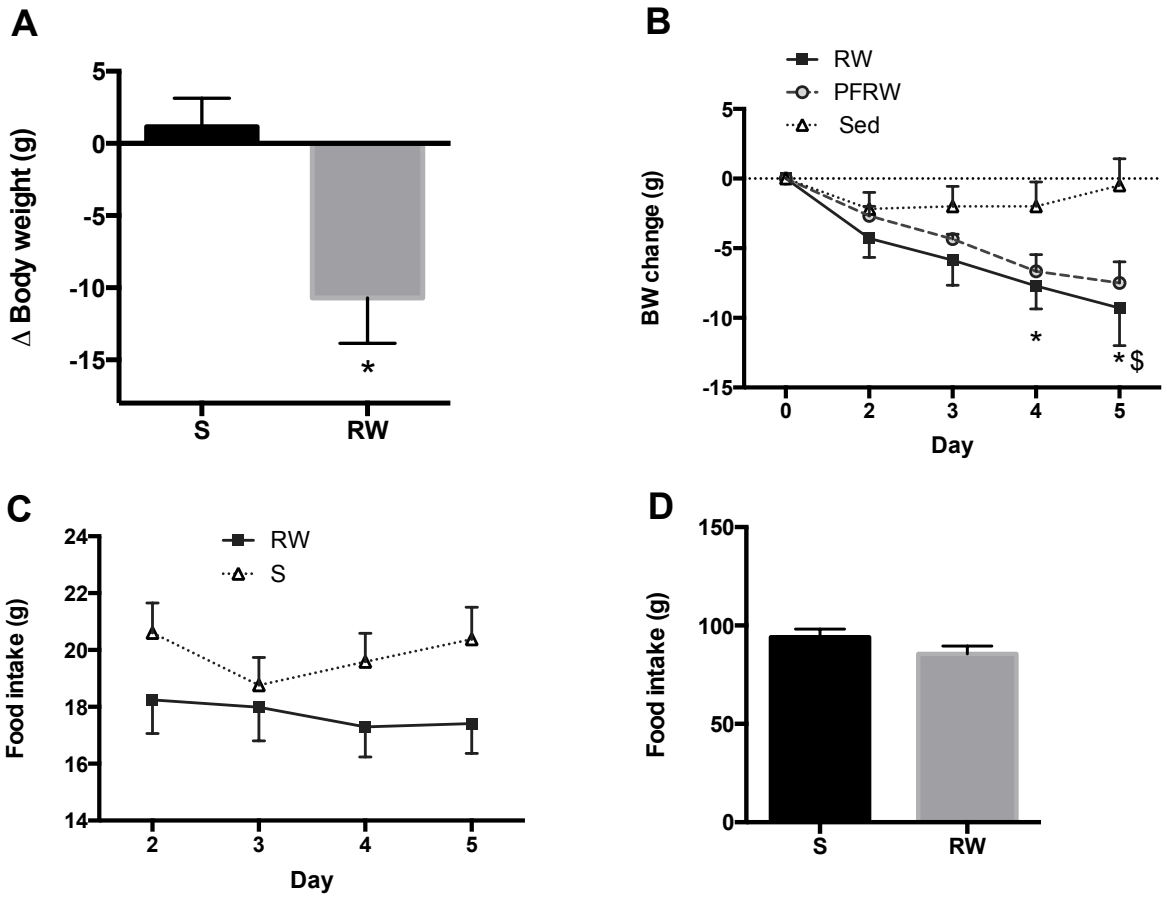

Figure 4.7 Exercise reduces body weight and tended to reduce feeding after five days of running wheel access.

By the fourth day of exercise, running wheel (RW) animals were in negative energy balance relative to baseline (A, B), and tended to eat less (C, D) compared with sedentary (S) controls. The effect of exercise treatment group on body weight depended on time (exercise x time $F_{8, 64}$=3.7, p<0.001) * RW vs S, $^{\$}$PFRW vs S; *p<0.05,* n=6-7.

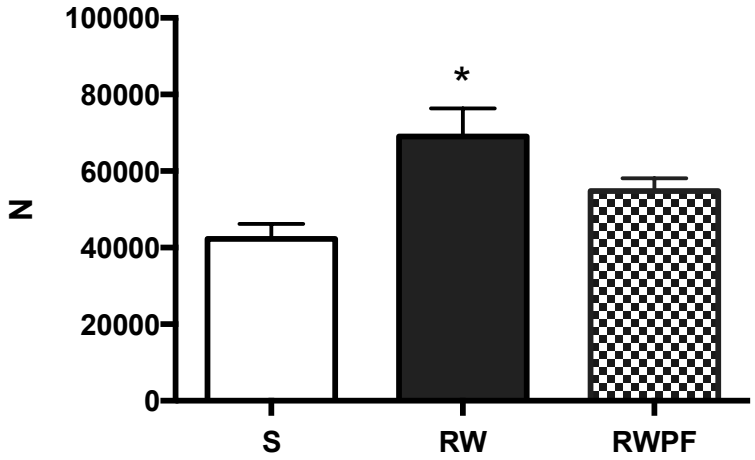

Figure 4.8 Voluntary running wheel exercise increases brain derived neurotrophic factor in the hypothalamic paraventricular nucleus.
There was a significant effect of treatment group on BDNF positive cells in PVN ($F_{2, 13}$=6.3, *p*=0.01). BDNF was significantly higher in RW compared with S. Neither RW nor S was different from PFRW; n=4-6.

Discussion

Our data show that exercise differentially affects BDNF over a short period of exercise training, but does not similarly affect BDNF in calorie restricted pair-fed animals, despite similar effects on energy balance. After long-term exercise training (eight weeks) exercise had no effect on PVN BDNF. These data imply that BDNF may be relevant during early stages of an exercise program for fostering homeostasis to maintain the new lower body weight. Several lines of evidence suggest wheel running and BDNF may have be involved in a common pathway regulating the effects of exercise on feeding. In the hypothalamus, volitional running and BDNF have anorexigenic effects through seemingly similar mechanisms. Both wheel running and BDNF increase corticotrophin releasing

hormone (CRH) in the PVN (Jeanneteau, Lambert et al. 2012) (Toriya, Maekawa et al. 2010) (Cao, Choi et al. 2011), and antagonizing corticotrophin releasing hormone receptors 1 and 2 (CRH R1 and R2) abolishes BDNF and running wheel effects on food intake and body weight (Toriya, Maekawa et al. 2010). Additionally, BDNF is essential for melanocortin receptor 4 (MC4R) anorexigenic effects (Xu, Goulding et al. 2003) (Nicholson, Peter et al. 2007) and in the absence of MC4R, wheel running attenuates hyperphagia and body weight gain (Irani, Xiang et al. 2005; Haskell-Luevano, Schaub et al. 2009). In other words, exercise and BDNF both reduce MC4R knockout-associated hyperphagia and obesity. Wheel running is a component of environmental enrichment, and it has recently been suggested that environmental enrichment, including wheel running, alters food intake and adiposity via a hypothalamic BDNF-mediated mechanism (Cao, Choi et al. 2011).

The effects of exercise to promote obesity resistance appear to be sustained even after stopping RW access (Patterson, Bouret et al. 2009), implicating that exercise causes long-term effects. After six weeks, however, the pair-fed animals began to gain weight such that by the end of the study they were positive energy balance relative to baseline and had gained weight compared with running wheel and treadmill groups. This indicates that changes in feeding may adapt over time, or that the effects of exercise on appetite may be temporary, though improvements in body composition, as indicated by the fat/lean mass ratio, are sustained. Conversely, previous reports have identified that three weeks of exercise confers long term maintenance of reduced body weights post exercise, but BDNF expression in the PVN was reportedly not elevated in running wheel compared with sedentary animals at the three week time point (Patterson 2007). This study measured gene expression, however it is possible that during wheel running BDNF expression is post-transcriptionally regulated. Additionally, BDNF is capable of retrograde and anterograde transport (Nawa, Carnahan et al. 1995; Altar, Cai et al. 1997; Conner, Lauterborn et al.

1997) and the possibility that BDNF originally expressed in extra-hypothalamic areas is trafficked to the hypothalamus during wheel running cannot be overlooked. Future studies are needed to determine whether our observation that BDNF is elevated during the first week of running wheel exercise is relevant to the observed reductions in feeding and improvements in body composition.

We report that eight weeks of exercise training prevented overfeeding and conferred significant improvements in body composition in adult SD rats. Furthermore, exercise-induced preventions in weight gain were, in part, a direct cause of the exercise-induced feeding reduction. Our control group was comprised of sedentary rats singly housed in standard shoebox cages. Therefore, these data can be interpreted in two ways. One is to suggest that exercise is beneficial to maintaining energy homeostasis, and the other is to say that being sedentary leads to aberrant regulation of energy balance in conditions where food is made available ad libitum. Recent human data supports both interpretations. In support of the former, in obese adolescents an acute bout of high intensity exercise training reduced 24-hour food intake (Thivel, Isacco et al. 2012) in amounts to confer negative energy balance without affecting energy expenditure (Thivel, Isacco et al. 2011; Thivel, Isacco et al. 2012). In support of the latter, subjects confined to complete bed-rest reportedly eat more food compared with those on a moderate exercise program (Bergouignan, Momken et al. 2010). Similarly, obese adolescents ate a larger post session meal when the session was comprised of bed rest compared with when they were freely moving about or freely moving and were given an exercise session (Thivel, Metz et al. 2013).

Similar to previous reports which suggest that hypothalamic BDNF expression is unchanged after eight weeks of running wheel access (Haskell-Luevano, Schaub et al. 2009), we did not observe differences in the number of BDNF positive cells in the PVN after eight weeks of exercise. We also did not

observe elevated $trkB_{ir}$ fibers in and around the PVN with running wheel exercise. Conversely, after eight weeks, running wheel access was associated with reduced density of $trkB_{ir}$ fibers in the PVN compared with treadmill-exercised animals. In our study, running wheel exercise conferred the greatest improvements in body composition compared with the treadmill exercise. Though future studies are needed to fully characterize these fibers and their function, $trkB_{ir}$ fibers might influence the excitability of PVN neurons. Wheel running reportedly activates the sympathetic nervous system and hypothalamic pituitary adrenal (HPA) axis (Girard and Garland 2002; Droste, Gesing et al. 2003; Droste, Chandramohan et al. 2007), resulting in increased epinephrine and glucocorticoid production, respectively. The HPA axis is initiated by corticotropin releasing hormone (CRH) produced by neurons of the paraventricular hypothalamus (PVN) (Sawchenko, Swanson et al. 1984). BDNF has been reported to increase corticotropin releasing hormone (CRH) in the PVN (Jeanneteau, Lambert et al. 2012) (Toriya, Maekawa et al. 2010)), and antagonizing CRHR1 and R2 receptors abolishes BDNF effects on food intake, body weight, and body temperature (Toriya, Maekawa et al. 2010). Thus a plausible mechanism is that during the initial stages of exercise, increased BDNF modulates the excitability of the PVN increasing activity of sympathetic nervous system and CRH, which is anorexigenic when acting on receptors in the brain. Chronic activation of the HPA axis can result in glucocorticoid excess, which is associated with central adiposity (Rosmond, Dallman et al. 1998), insulin resistance, hyperlipidemia, and increased glucose production (Saruta, Suzuki et al. 1986). Exercise, however, is associated with improved blood glucose regulation, reduced central adiposity (Giannopoulou, Fernhall et al. 2005; Giannopoulou, Ploutz-Snyder et al. 2005), and reduced insulin resistance (Richter, Garetto et al. 1982). It has been reported that HPA axis activity adapts after several weeks of exercise, which may explain why hyperglucocorticoidemia is not observed with chronic exercise (Fediuc,

Campbell et al. 2006). It is possible that, during exercise, BDNF plays a role in modulating activity of the HPA axis, since both wheel-running (Kawaguchi, Scott et al. 2005) and PVN BDNF injections (Toriya, Maekawa et al. 2010) have been reported to reduce feeding via a CRH-receptor mediated pathway.

The most robust improvements were observed in animals with voluntary running wheel access, compared with animals forced to run on a treadmill at during specific times of the day, despite similar running distances covered by treadmill and running wheel-exercised animals. Oddly, we did not observe correlations between changes in body composition and distances run on the wheel. Shapiro et al have previously reported that animals with access to volitional running reduced their caloric intake, even when animals ran a small amount (as little as 9 revolutions per day) (Shapiro, Cheng et al. 2011). They also found that this small interaction with the wheel enhanced leptin signaling in the ventral tegmental area, suggesting a possible hedonic substitution of the wheel for food (Shapiro, Cheng et al. 2011). Wheel running is often used as a component of environmental enrichment, and it has recently been suggested that environmental enrichment, including wheel running, alters food intake and adiposity via a hypothalamic BDNF-mediated mechanism (Cao, Choi et al. 2011). Our results showing that BDNF is elevated in the PVN of the animals with running wheels are consistent with this hypothesis, however it would be interesting to also see whether treadmill running similarly affects PVN BDNF during the first week of exercise training. It is possible that differences in body composition between treadmill and running wheel exercised animals can be explained by the coordinated movements necessary for each activity, or due to the 24-hour availability of the wheel compared with exercise given in one single dose each day (as it was for the treadmill group). In support of this possibility, one recent study in humans found that intermittent exercise (5 minutes 12 times/day) was associated with greater satiety and decreased hunger compared with continuous exercise (1 hour a day) (Holmstrup, Fairchild et al. 2013). Further

studies will be needed to elucidate which component of running wheel exercise confers the most benefit to body composition changes.

Perspectives and significance

The implications of these data highlight the importance of aerobic exercise as a tool for altering future behavior leading to weight status. Animals who ran ate less food than those than those that were sedentary, despite the higher energy demands of the exercise. This works in opposition to homeostatic theories of energy balance, thus we contend that it is likely that exercise alters plasticity of brain regions involved in the homeostatic control of energy balance. We report that exercise indeed elevates BDNF in the hypothalamic PVN, but these changes happen early during exercise training and are not present during later time points. After 6 weeks of exercise, most of the animals were approaching energy balance and were no longer eating to maintain a deficit. Still, exercising animals resisted the fat gains that their sedentary counterparts accrued. This suggests that the effects of exercise on energy balance may be useful for body composition changes and maintenance of a lower body weight, but that the ability for exercise to promote a negative energy balance is short-lived. Future studies are needed to determine the role of BDNF in this process and to elucidate the neuroanatomy and function of $trkB_{ir}$ fibers surrounding the PVN.

Chapter 5

Oxytocin in the ventromedial hypothalamus reduces feeding and acutely increases energy expenditure

Introduction

Oxytocin has anti-obesity effects, and is currently being tested clinically for use in the treatment of obesity and type-2 diabetes (Ott, Finlayson et al. 2013; Zhang, Wu et al. 2013). Though the mechanism for oxytocin effects have not been fully characterized, an extensive body of work has demonstrated anti-obesity effects of oxytocin in rodents (Olson, Drutarosky et al. 1991; Deblon, Veyrat-Durebex et al. 2011; Zhang and Cai 2011; Morton, Thatcher et al. 2012; Zhang, Wu et al. 2013) as well as humans (Zhang, Wu et al. 2013). Behaviorally, central oxytocin has been reported to delay meal onset (Arletti, Benelli et al. 1990) and reduce intake of sweet foods (Lokrantz, Uvnas-Moberg et al. 1997; Olszewski, Klockars et al. 2010; Mullis, Kay et al. 2013). Additionally, previous studies implicate a physiological role for endogenous oxytocin in reducing meal size (Blouet, Jo et al. 2009; Yamashita, Takayanagi et al. 2013). In addition to feeding effects, central oxytocin has potent effects on energy metabolism. For example, low doses of intracerebroventricular (i.c.v.) oxytocin promotes weight loss in rats without affecting feeding by elevating fat oxidation in adipose tissue, whereas higher doses of i.c.v. oxytocin both reduces feeding and increases lipolysis (Deblon, Veyrat-Durebex et al. 2011). Conversely, animals deficient in either oxytocin or its receptor show reduced energy expenditure, in some cases with normal feeding (Amico, Vollmer et al. 2005; Kasahara, Takayanagi et al. 2007; Takayanagi, Kasahara et al. 2008; Camerino 2009).

The main sources of oxytocin in the brain are the magnocelluar and parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) and the supraoptic nuclei (Sokol, Zimmerman et al. 1976; Rosen, de Vries et al. 2008). In particular, PVN oxytocin production is essential to maintaining energy balance. This is illustrated by observations that SIM1 haploinsufficiency, which reduces PVN oxytocin expression by 80%, results in an obese hyperphagic phenotype, and is reversed by central oxytocin administration (Kublaoui, Gemelli et al. 2008). In the PVN, magnocellular neurons release oxytocin both somato-dendritically (Pow and Morris 1989) and via axon terminals, most of which project to the posterior pituitary (Swanson and Kuypers 1980) where oxytocin is released into peripheral circulation. Parvocellular oxytocin neurons send projections to median eminence, and additional central locations including spinal cord and brainstem (Swanson and Kuypers 1980; Rinaman 1998). Though still speculative, strong evidence exists implicating the nucleus of the solitary tract (NTS) as a site where oxytocin affects feeding and energy expenditure, however less well known are the contributions of hypothalamic sites, such as the ventromedial hypothalamic nucleus (VMN) (For review see (Blevins and Ho 2013)).

There is evidence to support that the VMN may be involved in oxytocin effects on energy balance: 1) the VMN, which contains a high percentage of oxytocin receptors (Tribollet, Dubois-Dauphin et al. 1992; Boccia, Petrusz et al. 2013), has a well-known role in the regulation of energy balance. 2) VMN lesions are associated with reduced sympathetic nervous system activity and delayed satiety leading to obesity (Vander Tuig, Knehans et al. 1982; Sakaguchi, Arase et al. 1988; Takahashi, Ishimaru et al. 1997). Similarly, the prominent characteristic of oxytocin deficiency in mice is reduced energy expenditure due to reduced sympathetic tone (Takayanagi, Kasahara et al. 2008; Camerino 2009; Kasahara, Sato et al. 2013). 3) Peripheral injections of oxytocin sufficient to induce negative energy balance are associated with elevated cFos activation in both the VMN

and NTS (Zhang and Cai 2011), suggesting that in addition to NTS, VMN may be an important site for oxytocin effects on energy balance. Despite reports of oxytocin signaling in the VMN, immunohistological analyses reveal that very few oxytocin fibers reach this region (Leng, Onaka et al. 2008). Two mechanisms have recently been identified for how oxytocin may activate its receptor in the VMN: 1) VMN oxytocin receptor may be activated by oxytocin released dendritically from magnocellular oxytocin neurons (Sabatier, Leng et al. 2013). Based on the proximity of oxytocin neurons to the third ventricle, it has been hypothesized that PVN oxytocin may act in a paracrine manner following dendritic release by entering the ventricular system prior to activating oxytocin receptor in more distal areas of the brain (Leng, Onaka et al. 2008; Striepens, Kendrick et al. 2011); and 2) the fiber plexus lateral to the VMN contains axonal-dendritic synapses where oxytocin has been identified in axon terminals (Griffin, Ferri-Kolwicz et al. 2010).

We hypothesized that oxytocin in the VMN is a negative regulator of energy balance acting both to reduce feeding and increase energy expenditure. Here we show that oxytocin reduces feeding and acutely elevates energy expenditure, and conclude that the VMN may be one site where oxytocin acts to regulate energy balance.

Methods

Animals

Adult male Sprague Dawley (SD) rats (Charles River, Wilmington, MA) were individually housed in cages and maintained on a 12:12-h light dark cycle (lights on at 0400). Rooms were maintained at 21-22°C. Animals had ad libitum access to water and standard chow (Harlan Teklad 8604) except where indicated. All

protocols were approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Medical Center and University of Minnesota prior to experimentation.

Stereotaxic surgery and placement verification

Rats were anesthetized with intraperitoneal Xylazine (Butler, Dublin, OH, 3.5 mg/kg) and Ketamine (Ketaset, Fort Dodge, IA, 20 mg/kg) and surgically implanted with bilateral 28-gauge stainless steel guide cannulae (Plastics One, Roanoke VA) placed 1mm above the target injection site in the VMN: 0.5 mm lateral, 2.5 posterior to bregma and 8.6 mm below the skull surface, according to Paxinos and Watson (Paxinos and Watson 2007). Animals were given 1 week to recover and at least 4 days of gentle handling and sham injections prior to experimentation. Placement was verified using an NPY test, as described previously (Wang, Bomberg et al. 2007). Placement was deemed correct if the animal consumed more than 2 g of chow within 1 hour after 100 pmol NPY. Animals who did not respond to NPY were excluded from the study. Since NPY increases feeding in sites other than the VMN, histological staining was performed on a subset of animals and placement was verified as described previously (Wang, Bomberg et al. 2007). Brain tissues were post-fixed in 10% formalin solution for 48 hours, cryostat sectioned at a thickness of 40 μm, mounted on gelatin coated slides, stained with 0.1% thionin and treated with an ethanol gradient (30-100%) and clearing agent (Electron Microscopy Sciences, Hatfield, PA). Placement was deemed correct if the injection site was within 0.25 mm radius from the targeted site. This distance was selected based on diffusion coefficients of the injection volume (Nicholson 1985) and our previous data, showing the diffusion radius of 0.5 μl of 0.5% pontamine blue dye (Wang, Bomberg et al. 2007). Data from animals with misplaced cannulae were excluded from the data analyses.

Drug and injections

Lyophilized oxytocin acetate and NPY were purchased from Bachem Americas, Inc (Torrance, CA) and rehydrated in artificial cerebrospinal fluid (aCSF). All doses injected in a volume of 0.5 µl over a period of 30 seconds (s), with the injector left in place for an additional 15 s to ensure full delivery. Animals were injected either unilaterally, or bilaterally, as indicated for each experiment.

Spontaneous physical activity (SPA) and indirect calorimetry

Acrylic 17 x 17 inch chambers were customized with the capability to simultaneously record energy expenditure and SPA. Two sets arrays were affixed to the cage in the x-y plane and one set was placed three inches above for measurement of vertical movement. SPA was defined as the sum of time spent ambulatory and time spent moving in the vertical plane. Stereotypic activity was defined as time spent moving within a defined space around the animal (3.25 × 3.25 inches) as measured by beam breaks (Perez-Leighton, Boland et al. 2013).

Indirect calorimetry data were collected using Oxymax Lab Animal Monitoring System from Columbus Instruments (Columbus, OH). Prior to testing, chambers were calibrated using a primary gas standard. The chamber was sealed and room air pumped through at a rate of 3.0-4.7 L/min depending on the weight of the rat. Gas exchange measurements were automatically recorded every 30 s throughout the 12-hr sampling period with the exception of a 5 minute 30 s interval every 14.5 minutes, wherein room air was sampled for reference calibration. Data were recorded as kcal/hr rates for each 30s interval. To calculate hourly energy expenditure the 30 s rates were converted to kcal/30 s interval. The 30 s intervals were then summed to total kcal/hr, excluding the

sampling period for gas calibration, thus kcal/hr actually represent kcal/hr interval or kcal/43.5 minutes. SPA and stereotypic activity was simultaneously recorded using customized infrared activity sensors (Med Associates, St Albans, VT) to detect horizontal and vertical movement as previously described (Teske, Levine et al. 2006). Total time spent moving (SPA) was calculated as the time spent moving in the horizontal + vertical direction. The first 30 minutes post-injection was excluded from activity and calorimetry data to account for potential confounds in activity due to handling during injections and to allow for the air in the calorimetry chambers to equilibrate after being sealed.

Calculation of resting energy expenditure (REE) and non-resting energy expenditure (NREE) components of total energy expenditure (TEE)

Energy expenditure measurements and SPA were collected at 30 s intervals as described above. REE was calculated by averaging the lowest 10 energy expenditure recordings (5 minutes) over the first two-hours post injection and verifying them against SPA measurements to be sure that these points reflected times where the animals were not moving. This was necessary because in some cases we were not able to find enough points of inactivity in the oxytocin-injected animals during the first hour post-injection to make the REE estimation. For validation of our methods, we compared the REE estimates generated from the data in the two-hours immediately post injection with REE estimates using data from the 12-hour testing period using the lowest 10 energy expenditure points. We did not observe unusually low estimations of energy expenditure around the time when room air was sampled, as others have reported previously (Gavini, Mukherjee et al. 2014), thus we did not omit the lowest five points (Gavini, Mukherjee et al. 2014). NREE was calculated as TEE over the first hour-REE. As a final validation, we compared REE and NREE from the same aCSF treated

animals that were used in two different experiments described below (Experiment 3 and Experiment 4).

Since each hour had three calibration cycles, where data points were missing for 5 minutes 30 s each cycle, it was necessary to extrapolate over that period to get an estimate of one hour TEE. We averaged the 30s energy expenditure measurements (kcal/hour) in each subject, and multiplied this by the total the number of 30s intervals missing due to calibration cycles:

Σ measured energy expenditure (kcal) for 43.5 min + average energy expenditure during 43.5 min * 33 (number of missing 30s intervals)= TEE.

NREE was calculated as estimated TEE-REE.

Conditioned taste aversion

We performed a two-bottle conditioned taste aversion test (Wang and Kotz 2002; Wang, Bomberg et al. 2007). The premise for this test is that when rats are exposed to saccharin and water simultaneously, they chow a preference for saccharin. When animals are exposed to saccharin after being given an injection of a drug with aversive properties, their preference for saccharin is markedly reduced during subsequent exposures. In this case, CTA was used to test whether oxytocin has aversive properties. Sixteen naïve SD rats were deprived of water for 23.5 hours and had scheduled water access for 30 minutes per day for 7 days. Rats were then randomized into three treatment groups and given 15 ml of 0.1% saccharin immediately followed by a VMN injection of artificial cerebrospinal fluid (aCSF), 0.1, or 1 nmol oxytocin. This conditioned stimulation was repeated once after 48 hours. After an additional 48 hours, animals were given a two-bottle choice of either water or 0.1 % saccharin and the change in bottle weight was recorded. After a 72-hour washout period, the two-bottle test

was repeated with the order of the bottles reversed. The purpose of repeating the experiment was to see account for a potential confound of place preference. Data are presented as the average of the two trials calculated as % of total fluid intake

((saccharin solution intake)/(saccharin solution intake+ water intake)) x 100

General experimental protocols

Experiment 1: Effect of oxytocin in the ventromedial hypothalamus on normal feeding.

Twelve adult SD rats weighing 550-850g were maintained on Research Diets control formula (D12450B) for 2 weeks prior to the onset of testing and for the duration of the experiment. This diet was chosen for compatibility with the BioDaq periodic food recording system (Research Diets Inc., New Brunswick, NJ), which was used for measuring food intake. Using a repeated measures design with a 72-hour washout period, rats were bilaterally injected with 0.1, 0.5, 1.0 nmol oxytocin per side or vehicle (artificial cerebrospinal fluid (aCSF)). Treatments were given in a randomly ordered Latin Square design. Injections were given 30 minutes prior to the onset of the dark cycle. Food was allowed ad libitum until one hour prior to injections and immediately post injection. Body weights were recorded at 0, 24 and 48 hours post injection. One rat was removed from the study due to illness and his data were excluded from the statistical analyses.

Experiment 2: Effect of oxytocin on deprivation-induced feeding

Twelve Naïve adult SD rats weighing 400-800g were individually housed in wire cages. Bilateral injections of oxytocin were given 3 hours into the light cycle after 16 hours of food deprivation at doses of 0 (aCSF), 0.1, and 1nmol per side. Repeated measures design was used with 72-hour washout between

treatments, as described in experiment 1. Food was made available immediately post-injection and food intake and spillage were measured at 1, 2, 4, 24 hours. Body weights were measured at baseline and 24 hours. Two animals were removed from the statistical analyses, one due to incorrect placement and one due to illness.

Experiment 3: Effect of oxytocin in the ventromedial hypothalamus on energy expenditure and spontaneous physical activity.

Eight SD rats weighing 550-950g were acclimated to customized 17 x 17 in. acrylic chambers until weight stable (5 days). Using a repeated measures design with 72-hour washout period, animals were unilaterally injected with 1 nmol oxytocin or vehicle (aCSF) 30 minutes before the start of the dark cycle. We switched to a unilateral design for the energy expenditure experiments because the calorimetry chambers take time to seal and calibrate and we wanted to minimize the amount of time required for making injections so that the variability in the timing of the injection for each animal relative to the timing of the light cycle could be minimized. Rats were placed inside calibrated 17x 17 in. indirect calorimetry chambers with perforated plastic flooring to allow for spillage collection. Food was removed one hour prior to injections and ample (ad libitum) standard chow was placed directly inside the cage immediately post injection. Water was available ad libitum. Energy expenditure and SPA were recorded for 12 hours post-injection during the dark cycle. Food, food spillage and body weights were measured prior to injections and at 12 hours when animals were removed from the chambers.

Experiment 4: Effect of oxytocin in the ventromedial hypothalamus on energy expenditure and spontaneous physical activity during fasting.

Experiment 3 was repeated using six rats only food was not made available during the testing period. One rat was removed from the study due to equipment failure.

Statistical analysis

Data analyzed using two-way repeated measures analysis of variance (ANOVA), one-way ANOVA or two-tailed paired t-tests were analyzed in Prism version 6.0 (GraphPad Software, Inc.). Since body weights were in some cases minimally different from test to test, analysis of covariance (ANCOVA) were performed for each hour of energy expenditure testing, with body weight as a covariate. For ANCOVA analyses, we used SPSS (Version 19.0. Armonk, NY: IBM Corp).

Results

Oxytocin in the ventromedial hypothalamus reduces normal feeding without causing taste aversion.

Oxytocin was given 30 minutes prior to the onset of the dark cycle, when rats would normally begin feeding. At the 0.5 and 1 nmol doses oxytocin reduced feeding during the first hour by 52 and 66% (food intake was 1.8 ± 0.5 and 1.3 ± 0.4 g for 0.5 and 1nmol doses, respectively, and 3.7 ± 0.7 for controls; $p<0.05$) **(Figure 5.1A)**. After 4 hours, animals injected with oxytocin had eaten significantly less than controls: 0.1 nmol reduced feeding by 27% (7.0 ± 1.1 g compared with 9.8 ± 1.2 g for controls), 0.5 nmol reduced feeding by 34% (6.4 ± 1.0 g) and 1 nmol reduced feeding by 22% (7.6 ± 0.8 g). Body weight change (-0.6 ± 1.6 g after 0.1 nmol oxytocin injections, -4.0 ± 2.7 g after 0.5 nmol injections and -1.9 ± 1.9 after 1 nmol injections compared with 0.2 ± 2.1 g for control animals; $p=0.5$, ns) and cumulative food intake (24.7 ± 1.3 g consumed after 0.1nmol injections, 25.4 ± 1.6 g after 0.5 nmol and 23.8 ± 1.1 g after 1 nmol compared with 27.6 ± 2.2 g consumed in the control group; $p=0.4$, ns) tended to be reduced at 24 hours post injection (figure not shown).

To determine whether oxytocin in the VMN reduces feeding due to taste aversion or malaise, we performed a two-bottle test as previously described (Wang and Kotz 2002; Wang, Bomberg et al. 2007). We observed that oxytocin reduced feeding at doses that did not cause conditioned taste aversion (1 nmol dose) **(Figure 5.1B)**. There was, however, a slight but significant reduced preference for saccharin in VMN oxytocin-injected animals at the 0.1 nmol dose compared with vehicle-injected controls. Animals injected with 0.1 nmol oxytocin still preferred saccharin to water (112.5 ± 7.6 g saccharin solution vs 22.6 ± 3.6 g water), though their preference for saccharin was less than aCSF injected animals (149.7 ± 12.3 g saccharin solution vs 11.0 ± 3.2 g water). Thus, the VMN may be one site where oxytocin reduces preference for non-nutritive sweet tasting foods, an effect that was previously reported in oxytocin knockout mice (Billings, Spero et al. 2006) and with oxytocin receptor antagonism (Olszewski, Klockars et al. 2010; Mullis, Kay et al. 2013).

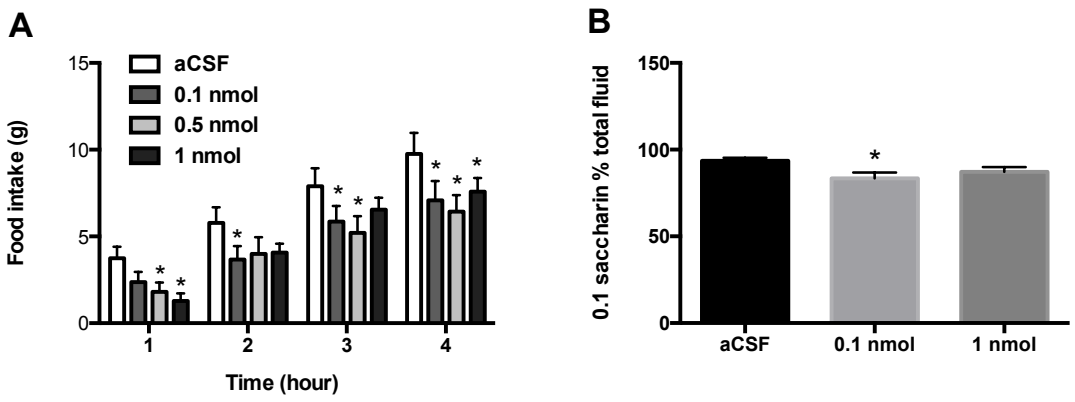

Figure 5.1 Oxytocin in the ventromedial hypothalamus reduces feeding without causing conditioned taste aversion

Food intake was significantly reduced by doses of 0.5 and 1 nmol oxytocin in the first hour post-injection. Food intake remained lower until up to 4 hours, where feeding was reduced by doses as low as 0.1 nmol (A). Oxytocin did not cause conditioned taste aversion, as over 50% of total fluid consumed in oxytocin-injected animals was saccharin (B) (84 and 88% of total solution was saccharin in 0.1 nmol and 1nmol groups, respectively). An oxytocin dose of 0.1 nmol in the VMN significantly reduced preference for saccharin (84% of total solution vs 93% for aCSF treated animals) and tended to reduce saccharin solution intake at the 1 nmol dose. Two-way repeated measures ANOVA with Dunnett's test for multiple comparisons (A) or one-way repeated measures ANOVA (B) *$p<0.05$ n=11 (A), n=6-7group (B).

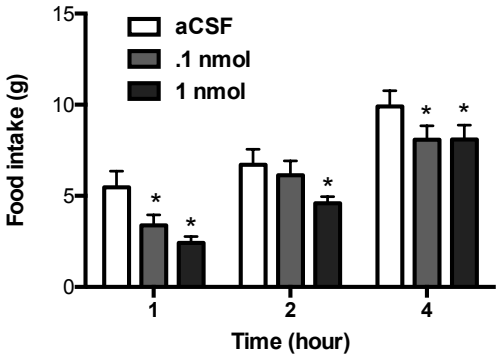

Figure 5.2 Oxytocin reduces feeding in animals fasted overnight. Bilateral injections of oxytocin were given 3 hours into the light cycle after 16 hours of food deprivation. Oxytocin reduced 1-hour food intake at doses of 0.1, and 1 nmol. After 4 hours, feeding was still significantly reduced after doses as low as 0.1 nmol. *Two-way repeated measures ANOVA with Dunnett's test for multiple comparisons. Statistical significance is compared with aCSF *p<0.05, n=10.*

Oxytocin in the VMN Reduces Deprivation-induced Feeding

After 16 hours of fasting, animals injected bilaterally with oxytocin at either 0.1 or 1 nmol ate 38 and 56 % less than controls in the first hour **(Figure 5.2)**. Control animals ate 5.0 ± 0.9 g during the first hour, compared with 3.6 ± 0.5 and 2.5 ± 0.3 g for animals injected with 0.1 and 1 nmol of oxytocin, respectively ($p<0.05$). By 4 hours feeding both groups still had a cumulative reduction in feeding by 18 %; control animals ate 9.3 ±0.8 g and animals injected with 0.1 and 1 nmol oxytocin ate 7.8 ±0.7 and 7.6 ±0.5g, respectively ($p<0.05$). After 24 hours, food intake (30.0 ± 1.6 g for aCSF, 27.7 ± 1.5 and 27.5 ± 1.6 g after 0.1 and 1 nmol injections, respectively) and body weight change (16.6 ± 2.2g for aCSF, 10.2 ± 3.3 and 9.2 ± 3.0 g after 0.1 and 1 nmol injections, respectively) tended to be lower in oxytocin-injected animals (p=0.2 (ns) for food intake, p=0.08 (ns) for

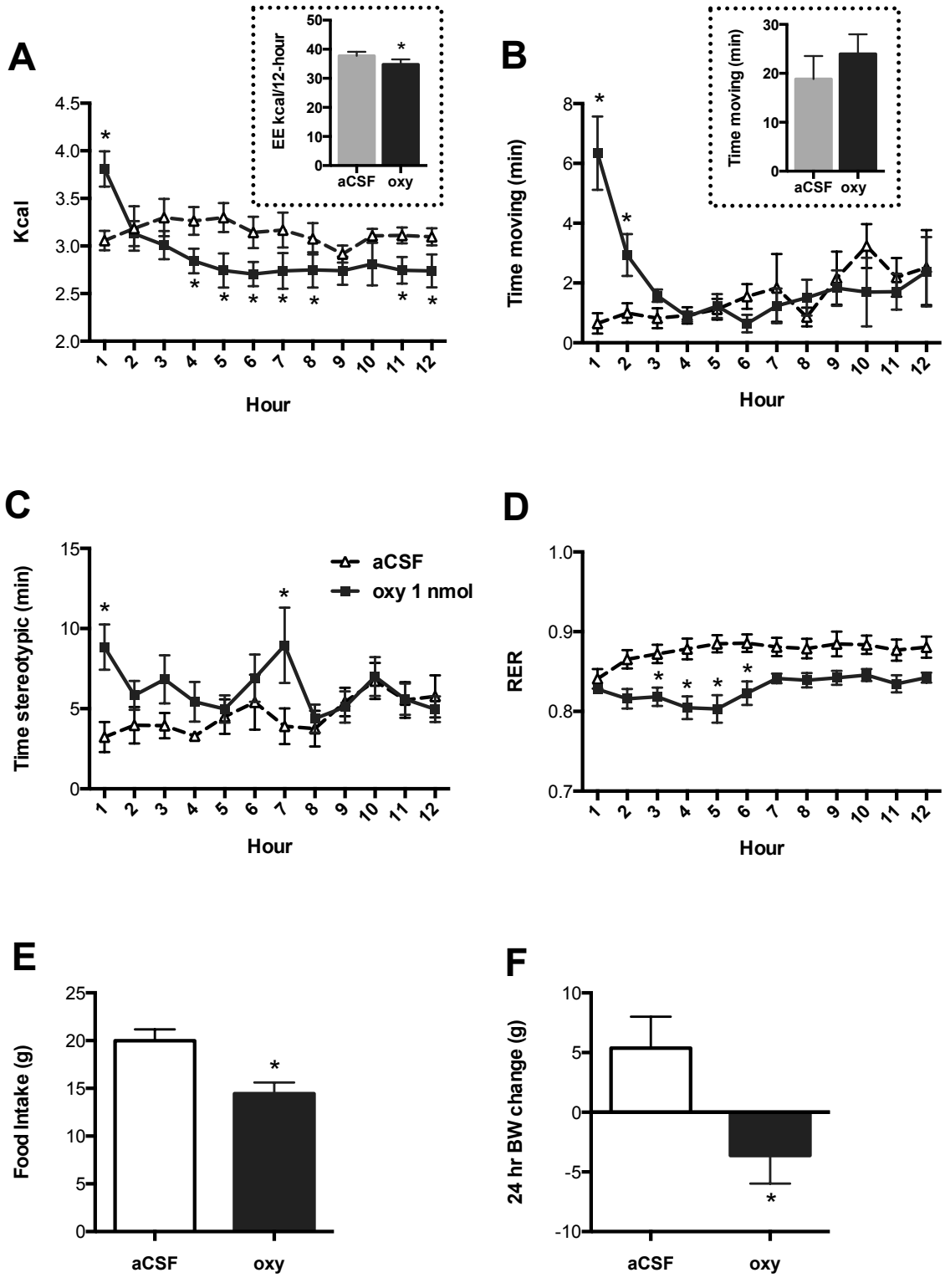
A
Kcal
Hour
EE kcal/12-hour
aCSF
oxy
B
Time moving (min)
Hour
Time moving (min)
aCSF
oxy
C
Time stereotypic (min)
Hour
aCSF
oxy 1 nmol
D
RER
Hour
E
Food Intake (g)
aCSF
oxy
F
24 hr BW change (g)
aCSF
oxy

Figure 5.3 Oxytocin in the ventromedial hypothalamus increases energy expenditure and SPA.

Oxytocin increases energy expenditure (A), spontaneous and stereotypic activity (C, D) during the first hour post injection, however energy expenditure remained lower for most of the remainder of the testing period leading to an overall reduction in 12-hour expenditure (A *inset*). Respiratory exchange ratio (RER) was reduced by oxytocin injections (B), possibly as a consequence of reduced feeding (E) which shifts nutrient utilization in favor of fat oxidation. Oxytocin injections reduced body weights during the 12-hour dark cycle (F). *ANCOVA using body weight as a covariate (A), two-way ANOVA (treatment x time) with Sidak's multiple comparisons test (B-D). Two-tailed paired t-test (E, F). N=8 (repeated measures) *p<0.05.*

body weight change) (figure not shown). Together, these data indicate 1) that oxytocin in the ventromedial hypothalamus acutely reduces deprivation-induced feeding without producing compensatory elevations in feeding during the 24 hours post-injection and 2) that oxytocin effects on feeding are present when the drug is given during the light cycle during food deprivation.

Oxytocin in the VMN acutely increases energy expenditure and SPA

We tested whether oxytocin increases energy expenditure in the VMN. Animals were injected 30 minutes prior to the onset of the dark cycle, when activity normally increases. We found acute elevations in total energy

expenditure due to VMN oxytocin injections **(Figure 5.3A)**. These effects were abolished after the first hour, and energy expenditure remained lower in the oxytocin animals for the remainder of the 12- hour testing period **(Figure 5.3A, inset)**. The elevation in energy expenditure during the first hour corresponded to increases in SPA **(5.3B)** and stereotypic activity **(5.3C)**. The effects of oxytocin on SPA were limited to the first hour post-injection **(5.3B)**. The respiratory exchange ratio (RER) was reduced in animals given oxytocin injections **(5.3D)**. In looking at the 12-hour cumulative food intake, we found that the oxytocin-injected animals ate significantly less than vehicle treated controls **(5.3E)**. Additionally, oxytocin injections significantly reduced body weight during the 12-hour dark cycle **(5.3F)**.

Effects of VMN oxytocin on energy expenditure and SPA without access to food

We tested whether differences in feeding could explain the reduced RER and some of the discrepancy between energy expenditure and activity levels observed in the time period after the first hour post-injection. As in the case where food was available during the testing period, oxytocin significantly increased energy expenditure during the first hour post-injection, but not thereafter **(Figure 5.4A).** There were no differences in 12-hour energy expenditure **(5.4A inset)**, indicating the effects of VMN oxytocin on energy expenditure are acute. Similarly, SPA was elevated during the first hour after oxytocin but not vehicle injections **(5.4B)**, however there were no differences in stereotypic activity when food was not available during the testing period **(5.4C)**. This indicates that the presence of food in the cage did not contribute to the elevations in activity level during the first hour. RER was reduced at a similar rate in both groups when neither group had access to food **(5.4D)**.

VMN oxytocin effects on resting (REE) and non-resting (NREE) components of energy expenditure

To validate our methods of calculating REE and NREE, we compared REE and NREE from aCSF treated animals over the two experiments, since the same animals were used during both sets of experiments. We found that there were no differences in REE or NREE in aCSF animals during Experiments 3 and 4 (**Figure 5.5**). Since elevations in total energy expenditure are mainly in the first hour, we compared REE and NREE during the first hour post-injection from experiments 3 and 4 (Fig. 5). Oxytocin increased REE, NREE and total energy expenditure when food was available during the testing period **(5.5A)**. When food was not in the cage, oxytocin increased energy expenditure by increasing NREE **(5.5B)**, but not REE.

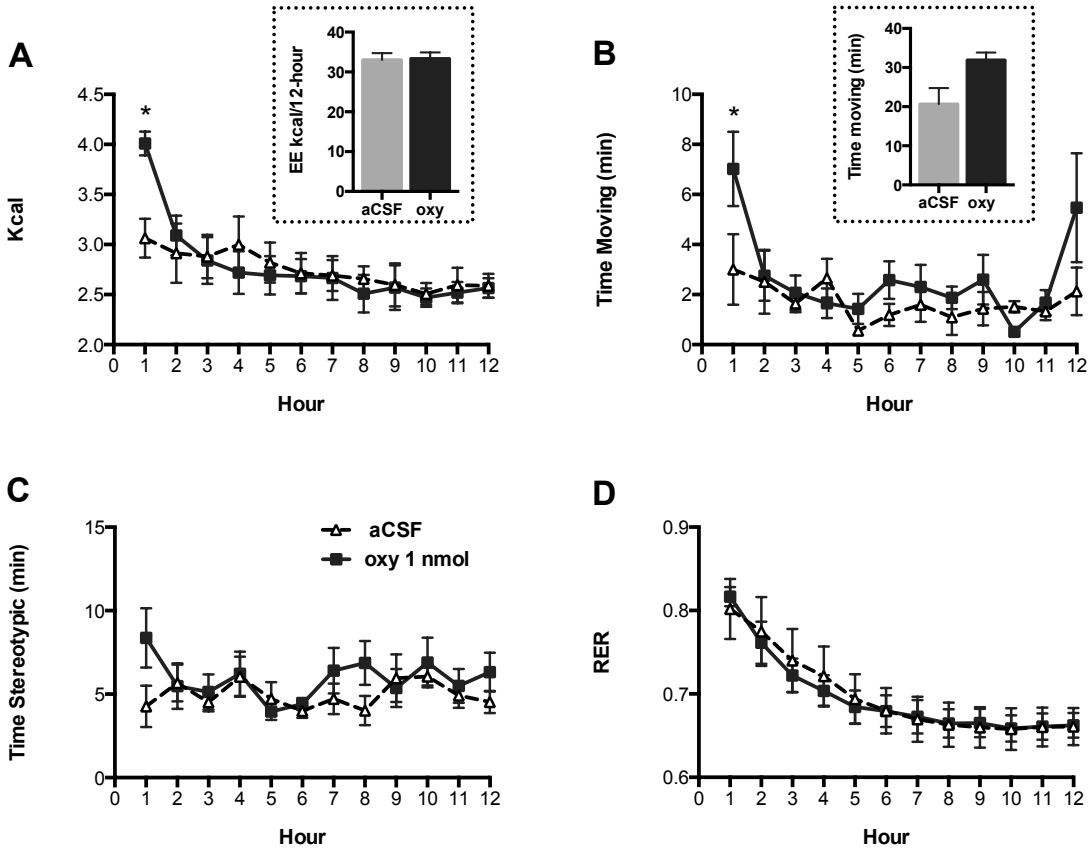

Figure 5.4 Oxytocin in the ventromedial hypothalamus increases energy expenditure and SPA during fasting.

Oxytocin increased energy expenditure during the first hour post-injection (A). There were no differences in 12-hour energy expenditure when food was not made available during the testing period (A *inset*). Respiratory exchange ratio (RER) was similarly reduced in both groups (B). SPA was elevated by oxytocin during the first hour (C), however there were no differences in stereotypic activity (D). *ANCOVA using body weight as a covariate (A),two-way ANOVA (treatment x time) with Sidak's multiple comparisons test (B-D). N=5 (repeated measures) *p<0.05.*

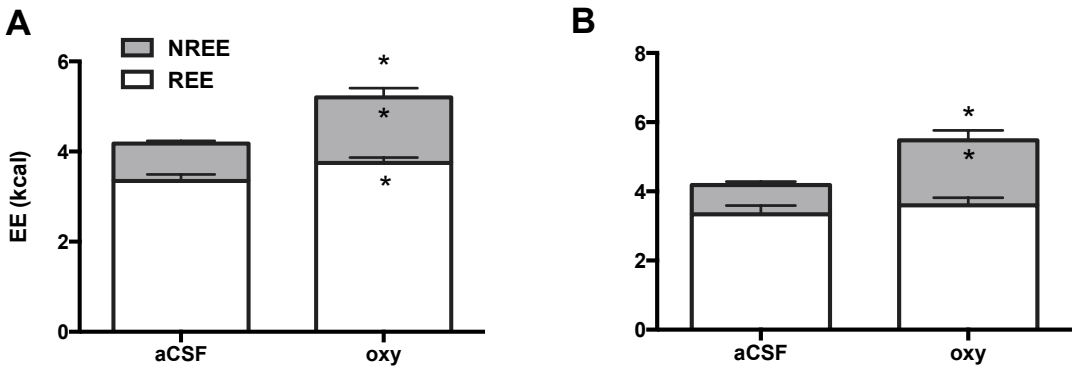

Figure 5.5 Oxytocin increases both resting and non-resting energy expenditure.

During non-fasted conditions, oxytocin increases resting energy expenditure (REE) and non-resting energy expenditure (NREE) for one hour immediately post-injection (n=8) (A). When food was not made available, only NREE was significantly increased by oxytocin in the hour post-injection (B). In both cases, oxytocin significantly increased total energy expenditure. *Two-tailed paired t test* *oxytocin vs. aCSF $p<0.05$.

Discussion

Our results identify the VMN as a novel site where oxytocin affects energy balance. To date, extensive work exists discussing the role of the hindbrain in oxytocin mediated satiety and energy expenditure (Olson, Drutarosky et al. 1991; Blevins, Eakin et al. 2003; Blevins, Schwartz et al. 2004; Peters, McDougall et al. 2008; Baskin, Kim et al. 2010; Morton, Thatcher et al. 2012; Uchoa, Zahm et al. 2013), however hypothalamic sites for oxytocin effects are less well known. In the NTS, oxytocin modulates the responsiveness to peripheral satiety signals such

as CCK (Olson, Drutarosky et al. 1991; Blevins, Eakin et al. 2003; Baskin, Kim et al. 2010) and leptin (Blevins, Schwartz et al. 2004; Wu, Xu et al. 2012). Though there is evidence for a central PVN-NTS pathway mediating the satiety promoting effects of leptin (Perello and Raingo 2013), effects of oxytocin on energy balance persist in leptin receptor-deficient rats (Maejima, Sedbazar et al. 2009; Morton, Thatcher et al. 2012), suggesting a leptin independent pathway. Peripheral oxytocin is associated with activation of the NTS, reduced feeding and weight loss in DIO and leptin receptor-deficient rats (Deblon, Veyrat-Durebex et al. 2011; Maejima, Iwasaki et al. 2011; Zhang and Cai 2011; Morton, Thatcher et al. 2012).

In addition to NTS, there is evidence for hypothalamic involvement in oxytocin effects on energy balance. Oleoylethanolamide (OEA), an ethanolamide produced by the small intestine after feeding, reduces food consumption in both fed (Gaetani, Oveisi et al. 2003) and fasted (Rodriguez de Fonseca, Navarro et al. 2001; Gaetani, Oveisi et al. 2003) rats via central oxytocin (Gaetani, Fu et al. 2010). While OEA activates the NTS neurons, recent evidence suggests that the role of NTS in OEA mediated satiety is via nor-adrenergic afferent input to PVN oxytocin neurons (Romano, Cassano et al. 2013). Gaetani et al reported that blocking oxytocin receptor in the third ventricle attenuated the anorexigenic effects of OEA without affecting NTS cFos activation (Gaetani, Fu et al. 2010), suggesting that oxytocin receptor activation in sites other than the NTS can also mediate feeding behavior. Additionally, Zhang et al. showed previously that the two primary sites activated by oxytocin were VMN and NTS (Zhang and Cai 2011), suggesting a potential role for VMN in oxytocin in feeding behavior. Herein, we report for the first time that oxytocin injections into the VMN reduce feeding in rats in both the fed and food-deprived state.

We used saccharin solution to perform a conditioned taste aversion test to ensure that oxytocin injected animals were not avoiding feeding due to malaise. We found that oxytocin did not cause a conditioned taste aversion (saccharin

solution still accounted for over 75% of fluid intake in oxytocin-injected animals), however saccharin intake was reduced in oxytocin-injected animals compared with aCSF (Figure 1B). Carbohydrate specific satiety has been reported using both oxytocin knockout (Amico, Vollmer et al. 2005; Miedlar, Rinaman et al. 2007; Sclafani, Rinaman et al. 2007) and wild type (Olszewski, Klockars et al. 2010; Mullis, Kay et al. 2013) mice. Previous studies have reported increased preference for both sugar and saccharin sweet solutions in oxytocin knockout mice (Billings, Spero et al. 2006). The significant reduction in saccharin solution observed in oxytocin-injected animals relative to aCSF-injected animals suggest that the VMN may be a site where oxytocin promotes satiety for sweet tastes.

A single unilateral oxytocin injection into the VMN acutely elevated energy expenditure, primarily by increasing activity **(Figure 5)**. The effects of oxytocin on energy expenditure and activity persisted for one hour, after which animals tended toward reduced energy expenditure without differences in SPA, leading to an overall significant reduction in energy expenditure for the 12-hour period (Figure 3A, C-D). For the energy expenditure experiments, in order to keep the timing of injections similar for each animal relative to the onset of the dark cycle while accounting for the extra time it takes to seal the calorimetry chambers, we did not perform bilateral injections. It is possible that we would have observed a longer duration of elevated energy expenditure, or greater increases had we done bilateral injections. Zhang et al observed elevated oxygen consumption that persisted for four hours following a single injection of oxytocin into the third ventricle (Zhang, Bai et al. 2011; Zhang and Cai 2011). A four-hour behavioral effect matches more closely with what we observed for the feeding effects of oxytocin, however future experiments are needed to investigate whether a longer duration of elevated energy expenditure occurs when both populations of VMN oxytocin receptors are stimulated. The difference in 12-hour energy expenditure was indeed attenuated when food was not available during testing. This suggests

that expenditure reductions in oxytocin-treated animals after the first hour post-injection observed when food was made available during testing were mainly due to oxytocin-induced feeding reductions and subsequent reduction in food related thermogenesis, not compensatory expenditure reductions.

We asked whether activity explained all of the difference in energy expenditure during the first hour post-injection, or whether oxytocin elevates resting energy expenditure (REE) as well as non-resting energy expenditure (NREE). We found that in the absence of food, NREE primarily contributed to the differences in energy expenditure, however when food was present both NREE and REE were elevated in the oxytocin-treated animals. It is interesting that in the presence of food oxytocin elevated REE during the first hour post-injection. It has been recently reported that endogenous oxytocin is required for diet-induced energy expenditure, thus mice lacking oxytocin neurons fed a high-fat diet were more susceptible to weight gain due to reduced energy expenditure (Wu, Xu et al. 2012). We observed elevated REE in animals fed standard chow that were given oxytocin. This may be related to thermic effect of food, however our oxytocin-treated animals also ate less food than aCSF treated controls. Alternatively, lack of differences in NREE between oxytocin-treated animals and controls in the absence of food may be due to a floor effect, since NREE was equally low for both animals when food was not made available during testing. In support of the former (that oxytocin in the VMN increases the thermic effect of food), recently it was reported that oxytocin in the DMH/VMN is essential for cold-induced thermogenesis (Kasahara, Sato et al. 2013). In addition to cold-induced thermogenesis, the metabolic activity of brown adipocytes, and in particular functioning uncoupling protein 1, is essential for diet-induced thermogenesis.(Feldmann, Golozoubova et al. 2009). Though oxytocin receptor deficient mice have normal expression of uncoupling protein 1, they have reduced β3-adrenergic receptor expression and elevated expression of α2A-

adrenergic receptor as well as larger fat depots in brown adipocytes (Kasahara, Sato et al. 2013). β3-adrenergic receptor and α2A-adrenergic receptor have opposing actions in brown adipose tissue, β3-adrenergic receptor activation increases thermogenesis whereas α2A-adrenergic receptor activation inhibits thermogenesis (for review see (Cannon and Nedergaard 2004)) (Kasahara, Sato et al. 2013). Our finding that REE was elevated in oxytocin-injected non-fasted animals may reflect increases in diet-induced thermogenesis, but more investigation is necessary to determine if this is the cause. In addition to elevating thermogenesis, peripheral β3-adrenergic receptor activation has been shown to reduce feeding (Tsujii and Bray 1998). Thus, the finding that oxytocin alters the expression of adrenergic receptors in brown adipose tissue raises the question as to whether oxytocin in the VMN reduces feeding indirectly, via activation of the sympathetic nervous system. This would explain why animals lacking oxytocin receptor or deficient in oxytocin have normal food intake (Amico, Vollmer et al. 2005; Kasahara, Takayanagi et al. 2007; Takayanagi, Kasahara et al. 2008; Camerino 2009), whereas animals injected with oxytocin have reduced feeding and increased energy expenditure.

PVN oxytocin neurons have previously been shown to exhibit diurnal rhythmicity in oxytocin expression, whereby oxytocin expression is increased during the day and reduced at night (Zhang and Cai 2011). Additionally, oxytocin neurons also are affected by energy status. Fasting reduces PVN oxytocin production (Kublaoui, Gemelli et al. 2008; Tung, Ma et al. 2008; Flak, Jankord et al. 2011; Blevins and Ho 2013) and re-feeding increases oxytocin production (Kublaoui, Gemelli et al. 2008). The current data show that oxytocin injections reduced deprivation-induced feeding **(Figure 5.2)** three hours after the onset of the light cycle. Similarly, oxytocin significantly reduced normal feeding and increased energy expenditure and activity at the onset of the dark cycle. Thus, in the VMN the effects of oxytocin may not be limited by circadian or behavioral

influences on signaling at the receptor level, however since our rats were injected in two different conditions (fed vs fasted) during different times of the day future experiments are necessary to determine circadian effects of oxytocin injections in both the fed and fasted state. Our finding that oxytocin reduces 12-hour energy expenditure when food is available during testing differ from Zhang et al., who reported that ventricular oxytocin injection during the dark cycle did not affect energy expenditure during the dark cycle (Zhang and Cai 2011). One possible reason for this discrepancy is that Zhang et al maintained their mice on a high-fat diet, whereas our rats were on standard chow. As mentioned previously, it has been shown that oxytocin increases diet-induced energy expenditure and in the context of a high-fat diet the increases in expenditure are associated with reduced body weight whereas at a chow diet they are not (Wu, Xu et al. 2012). Thus it is possible that in the context of a high fat diet there are no differences in 12-hour energy expenditure due to increased thermic effect of food due to oxytocin injections, despite an overall caloric reduction. Conversely, the thermic elevations in thermic effect of food are not great enough to overcome energy expenditure deficits due to reduced feeding on standard chow.

Our study has some limitations. One is the timing of injections during the fed and food-deprived state. Fasting oxytocin injections were performed during the inactive period and non-fasting injections were performed at the onset of the dark cycle, when animals would normally eat their first meal. Because oxytocin receptor may also have some circadian rhythmicity, we cannot say from our data whether oxytocin would be similarly anorexigenic in fasted animals that were injected at the onset of the dark cycle. Another limitation of this study is that oxytocin can act as an agonist to the vasopressin V1A receptor (Manning, Misicka et al. 2012), which is also present in hypothalamus; thus the present data do not distinguish whether oxytocin in the VMN exerts energy regulatory effects via the oxytocin or vasopressin receptor. Future experiments are required to

decipher which receptor population exogenous oxytocin acts to alter feeding, activity, and energy expenditure.

In summary, direct injections of oxytocin in the VMN reduce feeding and elevate energy expenditure during both the fed and fasted state, implicating the VMN as a site where oxytocin has anti-obesity effects. It remains to be determined whether the VMN plays a role in endogenous effects of oxytocin on energy balance. Based on the current dataset showing this is worth further investigation.

Perspectives and significance

Oxytocin is in the beginning stages of clinical use for the treatment of obesity and diabetes (Ott, Finlayson et al. 2013; Zhang, Wu et al. 2013), though we do not fully understand the mechanism of how oxytocin promotes a negative energy balance, nor have we characterized the sites of oxytocin effects. Understanding the site and mechanism of oxytocin effects on feeding and energy expenditure will help in developing targeted therapeutics. In some cases, oxytocin is being used to treat non-metabolic disorders, such as autism and schizophrenia, where anorexigenic effects or elevated energy metabolism might be undesirable. In other cases, genetic disorders leading to obesity, such as Prader Willi syndrome where oxytocin neurons are reduced (Swaab, Purba et al. 1995), or polyphorphisms of the fat mass and obesity-associated (FTO) gene, which regulates oxytocin expression (Olszewski, Fredriksson et al. 2011) , knowing the sites and mechanism of oxytocin effects could help with the development of targeted delivery systems. Our data indicate that in addition to the NTS, the VMN is a central location where oxytocin may exert anti-obesity effects.

Chapter 6
General conclusions and future directions

The relevance of the effects of exercise on appetite in promoting adiposity resistance suggests that exercise may be an important tool for obesity treatment by tempering caloric intake in sedentary individuals. I show that exercise results in a temporary reduction in cumulative food intake that last approximately four weeks, followed by eating to maintain energy balance for at least eight weeks, whereas being sedentary is associated with eating for positive energy balance. During the early stages of exercise training, while animals were in negative energy balance, PVN BDNF is elevated in relationship to the amount of running performed by the animals. I did not observe significant changes in BDNF at the eight-week time point, suggesting that exercise may result in early plasticity changes in the PVN, which may alter the function or responsiveness of the PVN during the long-term. Though this study was mainly observational and no mechanistic conclusions can be drawn about BDNF in the PVN during exercise, these data provide insight into promising areas of future study. The next step in determining whether PVN BDNF has a central role in the anorexigenic response to exercise would be to block either BDNF or trkB receptor signaling during this early period of exercise and see whether the anorexigenic response persists. It

has previously been shown that stress paradigms increase expression of BDNF mRNA in the PVN (Rage, Givalois et al. 2002), which is associated with decreased inhibitory synaptic input leading to activation of PVN neurons (Verkuyl, Hemby et al. 2004; Verkuyl, Karst et al. 2005). It has been reported that BDNF reduces inhibitory signaling on PVN neurons via the removal of $GABA_A$ receptor from the post-synaptic membrane, leading to a more responsive PVN stress response (Hewitt 2006). It is tempting to speculate that the presence of trkB receptors on fibers surrounding the PVN area is also related to modulation of neurons involved in responsiveness to stress. In order to fully characterize the trkB immunoreactive fibers, tract tracing studies would need to be successfully undertaken in both retrograde and anterograde directions. A more detailed time course of trkB immunoreactivity around the PVN would need to be explored during both volitional and forced running paradigms, and correlated to measurements of PVN associated hormonal responses, in order to determine the relevance of trkB immunoreactive fiber density to PVN activity.

The data within this dissertation shows that oxytocin reduces feeding and increases both activity and energy expenditure in the VMN. These data are relevant to understanding mechanisms by which oxytocin reduces feeding and provide insight into the role of oxytocin in the central regulation of energy balance. Although PVN oxytocin production has been demonstrated to be essential to maintaining energy balance (Kublaoui, Gemelli et al. 2008), the

potential sources of endogenous oxytocin to the VMN are a mystery. Despite reports of oxytocin signaling in the VMN, immunohistological analyses reveal that very few oxytocin fibers reach this region (Leng, Onaka et al. 2008). Thus the endogenous relevance of our finding, that oxytocin in the VMN reduces feeding and increases activity and energy expenditure, as well as the relevant circuitry needs to be validated. Recent data have shown that VMN oxytocin receptor may be activated by oxytocin released dendritically from magnocellular oxytocin neurons (Sabatier, Leng et al. 2013). Alternatively, the fiber plexus lateral to the VMN contains axonal-dendritic synapses where oxytocin has been identified in axon terminals (Griffin, Ferri-Kolwicz et al. 2010), thus PVN connections may directly contact VMN dendrites, but this remains to be determined.

In summary, this dissertation offers the following conclusions relevant to the obesity research 1) that exercise alters plasticity mechanisms associated with the central regulation of feeding and promotes maintenance of a new lower body weight via altering appetitive responses, 2) that a population of trkB immunoreactive fibers exists in the area surrounding the PVN, though their function and characterization are unknown, 3) that volitional exercise is associated with early elevations PVN BDNF and 4) that oxytocin in the VMN promotes weight loss via reducing food consumption and elevating activity and energy expenditure.

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